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JANUARY, 1932

## THE MINERAL EXCHANGES OF MAN

II. EFFECT OF EXCESS POTASSIUM AND OF  
CALCIUM ON TWO NORMAL MEN AND  
ON AN OEDEMATOUS NEPHRITIC\*

By

SAMUEL H. BASSETT, C. A. ELLEN, AND W. S. McCANN

*(From the Department of Medicine, University of Rochester School of  
Medicine and Dentistry, and the Medical Clinic of the  
Strong Memorial Hospital, Rochester, N. Y.)*

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THE manner in which the body marshals its defenses against the excessive ingestion of inorganic acid radicals has been demonstrated by the results of several investigations (15, 35, 19, 14). There is less unanimity of opinion however in regard to the mechanism of the action of such cations as potassium and calcium.

In 1873 Bunge (11) attempted to explain the desire of herbivorous animals for common salt on the basis of excessive amounts of potassium in their diets. He showed that the diet of the ox contained from three to nine equivalents of potassium for each equivalent of sodium, whereas the diet of a carnivorous animal such as the cat contained not more than two equivalents of potassium for each equivalent of sodium. In the case of herbivora, he assumed that this ingestion of large amounts of potassium tended to raise the potassium content of the body fluids above the normal level with the result that the excess potassium was rapidly excreted by the kidney. He further postulated that during the period of transportation of potassium by the blood there was some exchange of anions between sodium and potassium salts. This exchange of anions led to an increase of sodium phosphate, sodium bicarbonate, etc., in the body fluids so that these substances were also present in amounts exceeding their renal threshold, hence sodium was excreted in the urine. Because of the considerable amount of sodium chloride normally present in blood, the anions

\* Aided by a generous grant of money from the Fluid Research Fund contributed by the Rockefeller Foundation.



derived from sodium salts by potassium were chiefly chlorine ions and these were excreted in combination with potassium. The end result was an excessive excretion of both sodium and chlorine.

As proof of this hypothesis he cited several experiments performed upon himself. After following the excretion of sodium, potassium and chlorine in his urine for several days, during which he lived on a carefully controlled diet, he took a very large dose of dipotassium phosphate (equivalent to 12.7 gm. of K) and noted a marked increase in the excretion of sodium, potassium and chlorine in the urine over a period of 24 hours. On the following day, when no potassium salt was added to his diet, the urinary sodium and chlorine excretion was distinctly less than during the control period. In a subsequent experiment an equivalent dose of potassium citrate produced identical effects upon the excretion of sodium and chlorine.

Meyer and Cohn (22) in 1911 noted that infants increased in weight when 0.4 to 0.7 per cent of the total weight of their diets consisted of sodium chloride or disodium phosphate. Weight loss occurred when the sodium salt was replaced by potassium or calcium salts. Several balance experiments were carried out during two-day periods. The intake and output of calcium, sodium, potassium, phosphorus and chlorine was observed on a constant diet, and the effect on the balance determined, following addition of sodium chloride, potassium bicarbonate, and calcium chloride and acetate to this diet. Of these salts sodium chloride had the least effect on the balance of the other cations and anions studied, while potassium bicarbonate produced an unfavorable effect on the balance of all but potassium. Calcium chloride which may be regarded as an acid-forming salt (17, 14) produced a distinctly unfavorable effect on the balances of both sodium and potassium.

The experiments of Bunge and Meyer and Cohn were of too short duration to determine whether this mobilization and excretion of minerals could be long enough continued to bring about any serious depletion of the mineral stores of the body.

Miller (23) followed the excretion of sodium and chlorine in the urine of pigs fed on a diet consisting chiefly of starch and noted that the addition of 11 to 14 grams of potassium acetate or phosphate to the daily diet produced an immediate increase in the excretion of sodium and chlorine in the urine. The effect was only temporary, and after a day or two of continued high potassium feeding the urinary excretion of sodium fell to a level even below that of the control period.

Following this (1926) he repeated these experiments on rats (24) and

determined the total excretion of calcium, phosphorus, sodium and chlorine, while the rats were on a constant diet, and again after the addition of a potassium salt to the basal ration. Observations were continued over an interval of about 2 weeks. The immediate effect was an excessive excretion of sodium and chlorine with a prompt return nearly to the basal level. There also appeared to be a slight increase in the excretion of both calcium and phosphorus while the animals were on the high potassium diet. Although a balance was not kept, Miller concluded that the increase in excretion of minerals during the high potassium feedings was too small to have a deleterious effect upon the mineral reserve of the animals.

Richards, Godden and Husband (27) (1924) observed the effect of adding large amounts of sodium chloride to the diets of growing pigs. An immediate increase in the potassium content of the urine was noted which was almost completely balanced by diminished fecal excretion of potassium. The effect on nitrogen, calcium and phosphorus retention was favorable. In later experiments (1927) (27) when potassium citrate was substituted for sodium chloride in the pig's diet, there was decreased assimilation of nitrogen, phosphorus and calcium. In respect to sodium the authors state in conclusion

The results obtained for growing pigs with regard to sodium excretion do not support the theory of sodium impoverishment put forward by Bunge as the result of experiments on himself.

Of the various potassium salts employed by different investigators the bicarbonate, acetate, citrate, phosphate and to a lesser degree, the chloride, have been found to produce immediate unfavorable effects upon the sodium balance, suggesting that the action of these salts is in some measure dependent upon the cation.

Opinion as to the mechanism of the action of calcium salts does not appear to be as uniform. Blum, Aubel and Hausknecht (7) maintain that the diuresis which sometimes follows the administration of calcium salts, is the result of direct antagonism between calcium and sodium and does not necessarily depend upon the nature of the anion. They cite an experiment performed upon an oedematous subject in whom diuresis was produced by alternate ingestion of calcium chloride and calcium lactate. At first small doses of these salts were effective but as the oedema diminished, increasingly large doses of salt became necessary to produce diuresis. The elimination of sodium, chlorine and potassium was followed in the urine. No correlation could be found between the excretion of potassium or chlorine and weight loss. On the other hand retention of sodium was

followed by increase in weight while excretion of sodium was associated with weight loss.

Haldane, Hill and Luck (17) assume that the diuretic action of calcium chloride is the result of an acidosis and Gambel, Ross and Tisdall (14) have shown that the acidosis is caused by the greater absorption of the chlorine ion than of the calcium ion. This is in accord with the work of Gamble, Blackfan and Hamflton (13) who believe the mechanism of the action of ammonium chloride and calcium chloride to be similar, both producing an acidosis. The ammonia-forming mechanism of the kidney (25) and the increase in acidity of the urine are not sufficient to compensate entirely for the hydrochloric acid formed from chlorine ingested, hence base is withdrawn from the blood and tissues, chiefly sodium and potassium. Results in agreement with these have been reported by Linder (19) following the administration of hydrochloric acid to normal men and to patients with nephritis.

In order to observe the effect of potassium upon the balances of the other cations in man, we have fed potassium citrate to two normal subjects and to a patient with nephritis and oedema. By way of comparison a known acid-forming salt—calcium chloride<sup>1</sup>—was then administered to the same subjects.

#### EXPERIMENTAL

The experiment was divided into three periods of 5 or 6 days<sup>2</sup> each, during which the balances of nitrogen, sodium, potassium, calcium, magnesium, iron, phosphorus and chlorine were determined.

The total dietary intake per period was computed from the mineral analysis of standard diets prepared in the metabolism kitchen. The excretion of nitrogen and minerals was determined from analyses of samples of urine and stool. (Methods of analysis, management of the metabolism units, etc., have been reported in a previous publication (4).)

During a preliminary or control period, only the standard diets were eaten by the subjects of the experiment. In the following periods a known

<sup>1</sup> In the following experiments it is to be regretted that more of the variable factors were not eliminated at the time the experiments were in progress. Subsequent analysis of the collected data has shown that accurate interpretation of the results obtained during the period of administration of calcium chloride was impossible owing to the variation in dosage of the calcium salt, the simultaneous administration of parathormone and lack of adequate control periods between the high potassium and calcium periods. The criticisms in regard to control periods and dosage are also applicable, but to a lesser degree, in the period in which potassium citrate was administered. Since it is difficult to separate completely that portion of the experiment which may be significant from that which is unimportant, and since it is hoped that our experience may prevent others from encountering similar pitfalls, the entire data have been presented.

<sup>2</sup> Results given in tables and charts have been computed on the basis of 5-day periods.

amount of potassium citrate or calcium chloride was taken in addition to the diet. The salts given were in solution and were administered in divided doses during the course of the day. Only the total amount of salt ingested during the period is recorded in the tables and charts. In each case the daily dose is approximately 1/5 of the total for the period.

Dietary and other factors which may influence the mineral metabolism have been summarized by Bauer, Albright and Aub (5). Briefly these are:

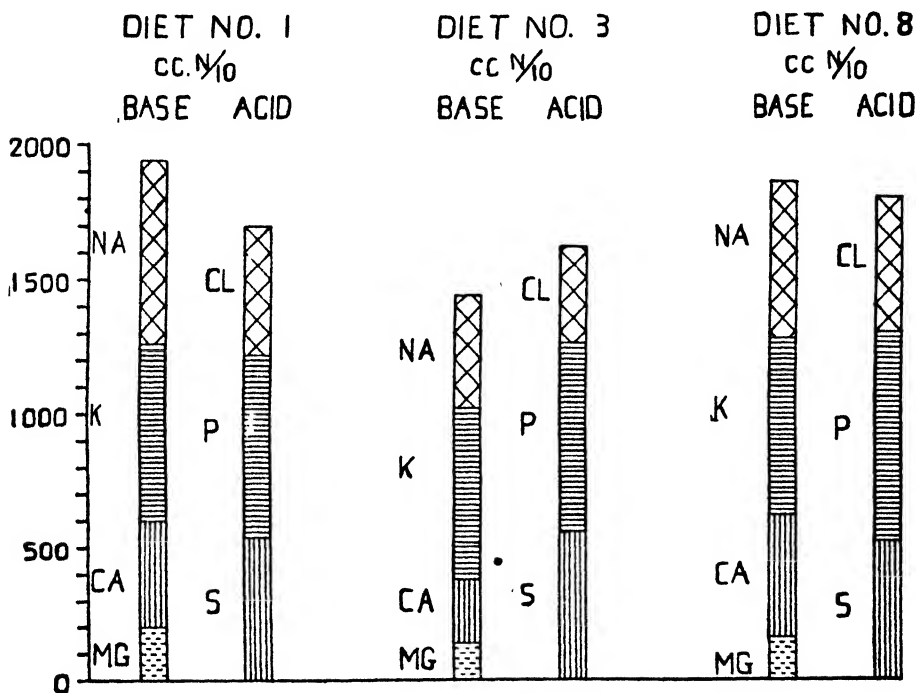


CHART 1. Acid and basic components of ash of three typical diets expressed in cc. of N/10 acid and base.

a.—level of mineral intake; b.—acid base values of diet; c.—relative amounts of presumably antagonistic cations in the diet; d.—accessory food factors—particularly vitamin D; e.—factors relative to age, weight, sex and activity of the subject.

A detailed description of the standard diets has been given elsewhere (4). It is believed that these diets were sufficient in respect to calories, protein, accessory food factors, and mineral content to maintain a normal healthy adult in mineral balance. In Chart 1 the acid base values of three typical examples of the ten diets used have been recorded graphically. The various acid and basic components have been expressed in terms of cubic

TABLE I  
NITROGEN AND MINERAL BALANCES IN TWO NORMAL SUBJECTS AND AN OEDEMATOUS NEPHRITIC. CONTROL PERIOD 5 DAYS

Element	Normal Subject Z Age 24 yrs. Wt. at beginning of period 57.4 kg. Wt. at end of period 56.8 kg.				Normal Subject S Age 23 yrs. Wt. at beginning of period 73.9 kg. Wt. at end of period 74.6 kg.				Subject M Nephritis Age 44 Wt. at beginning of period 75.8 kg. Wt. at end of period 75.9 kg.			
	Intake	Output		Balance	Intake	Output	Balance	Intake	Output	Balance		
Nitrogen	Food	Urine	75.0	-2.4	93.2	76.7	+11.9	52.6	41.3	+7.15		
		Stool	6.1			4.6			4.15			
	Total		81.1			81.3			45.45			
Phosphorus	Food	Urine	4.55	+0.47	10.07	5.78	+1.15	5.96	3.97	-0.43		
		Stool	3.62			3.14			2.42			
	Total		8.17			8.92			6.39			
Chlorine	Food	Urine	17.1	-5.17	13.66	12.88	+ 0.78	8.42	2.93	+5.49		
		Stool	—			—			—			
	Total		17.1			12.88			2.93			





Sodium	Food	— 13.82	Urine Stool	8.79 0.51		— 11.25	7.49 0.10	— 5.85	13.25 0.54
	Total	13.82		9.30	+4.52	11.25	7.59	+3.66	13.79
Potassium	Medication Food	14.22 26.66	Urine Stool	31.10 4.19		19.34 22.15	34.20 2.90	37.05 12.49	38.40 1.37
	Total	40.88		35.29	+5.59	41.49	37.10	+4.39	39.77
Calcium	Food	— 7.20	Urine Stool	1.01 6.53		— 6.30	0.93 5.16	— 3.44	0.16 3.14
	Total	7.20		7.54	-0.34	6.30	6.09	+0.21	3.30
Magnesium	Food	— 2.25	Urine Stool	0.73 1.52		— 1.81	0.67 1.11	— 0.98	0.19 0.50
	Total	2.25		2.25	0.00	1.81	1.78	+0.03	0.69
Iron	Food	— 0.143	Urine Stool	0.002 0.109		— 0.118	0.002 0.084	— 0.057	0.007 0.036
	Total	0.143		0.111	+0.032	0.118	0.086	+0.032	0.043
+0.014									

• In the normal subjects this period followed immediately after control period, in the nephritic subject it was preceded by CaCl<sub>2</sub> period.



TABLE III  
NITROGEN AND MINERAL BALANCES IN TWO NORMAL SUBJECTS AND AN OEDEMATOUS NEPHRITIC, CALCIUM CHLORIDE ADMINISTRATION\*  
Normal Subjects-6 Day Period Calculated to 5 Day Period; Nephritic Subject-5 Day Period

Element	Normal Subject Z Age 24 yrs. Wt. at beginning of period 57.9 kg. Wt. at end of period 57.0 kg. Ca in CaCl <sub>2</sub> = 17.8 gm. Cl <sub>2</sub> in CaCl <sub>2</sub> = 31.5 gm. 9.86 gm. CaCl <sub>2</sub> per diem Parathormone 100 Units on 5th Day of Period				Normal Subject S Age 23 yrs. Wt. at beginning of period 75.2 kg. Wt. at end of period 73.4 kg. Ca in CaCl <sub>2</sub> = 23.5 gm. Cl <sub>2</sub> in CaCl <sub>2</sub> = 41.70 gm. 13.04 gm. CaCl <sub>2</sub> per diem Parathormone 100 Units on 5th Day of Period				Subject M Nephritis Age 44 Yrs. Wt. at beginning of period 76.3 kg. Wt. at end of period 75.9 kg. Ca in CaCl <sub>2</sub> = 4.09 Cl <sub>2</sub> in CaCl <sub>2</sub> = 7.10 2.24 gm. CaCl <sub>2</sub> per diem. Parathormone 300 Units 100 Units on 1st, 3rd and 5th day of Period			
	Intake		Output		Balance	Intake	Output	Balance	Intake	Output	Balance	
Nitrogen	Food	— 92.1	Urine Stool	60.00 9.75		— 86.0	78.30 6.84		— 51.9	47.50 4.06		
	Total	92.1		69.75	+22.35	86.0	85.14	+0.86	51.9	51.56	+0.34	
Phosphorus	Food	— 10.14	Urine Stool	4.77 5.69		— 9.48	6.36 5.22		— 5.78	4.05 2.17		
	Total	10.14		10.46	-0.32	9.48	11.58	-2.10	5.78	6.22	-0.44	
Chlorine	Medication Food	31.5 13.77	Urine Stool	35.00 —		41.70 12.85	51.4 —		7.10 7.98	12.97 —		
	Total	45.27		35.00	+10.27	54.55	51.4	+3.15	15.08	12.97	+2.11	

Sodium	Food	— 10.65	Urine Stool	10.65 0.61		— 9.90	9.75 0.46		— 6.46	3.94 0.52	
	Total	10.65		11.26	-0.61	9.90	10.21	-0.31	6.46	4.46	+2.00
Potassium	Food	— 21.97	Urine Stool	21.50 3.93		— 20.20	26.00 3.69		— 12.89	10.90 1.58	
	Total	21.97		25.43	-3.46	20.20	29.69	-9.49	12.89	12.48	+0.41
Calcium	Medication Food	17.80 6.18	Urine Stool	3.21 18.80		23.50 5.64	3.43 23.90		4.09 3.70	0.27 10.30	
	Total	23.98		22.01	+1.97	29.14	27.33	+1.81	7.79	10.57	-2.78
Magnesium	Food	— 1.72	Urine Stool	0.61 1.29		— 1.60	0.76 1.20		— 1.03	0.45 0.77	
	Total	1.72		1.90	-0.18	1.60	1.96	-0.36	1.03	1.22	-0.19
Iron	Food	— 0.110	Urine Stool	0.002 0.067		— 0.096	0.002 0.085		— 0.062	0.002 0.071	
	Total	0.110		0.069	+0.041	0.096	0.087	+0.009	0.062	0.073	-0.011

\* Followed Potassium Citrate period for normal subjects; Control period for nephritic subject.

centimeters of N/10 acid and base. Phosphorus was considered di-basic (33). Inspection of the chart shows that there is no marked preponderance of acid over base or base over acid in the ash of the diets.

Of the three subjects studied, two were medical students aged 24, and 23 years respectively, in good health, who lived in the hospital during the period of observation, and took their meals in the metabolism kitchen. Their activity was in no wise restricted and they were permitted to have as much food and water as desired. In order to meet their energy requirements it was necessary to increase each article on the menu a uniform amount, to provide 50 and 75 per cent, respectively, more energy than was provided for in the original menus of the diets, which were calculated at a 2000 calorie basis.

The third subject was a man aged 44 years, ill with nephritis and oedema. His renal lesion was presumably primarily that of hemorrhagic Bright's disease with later development of secondary degenerative phenomena (1) (see case report). His activity was necessarily considerably restricted although he was not confined to bed but was permitted to be up in a wheel chair as much as he desired. The standard diets averaging about 2000 calories per diem proved adequate in his case.

All three subjects were essentially in nitrogen equilibrium or showed positive nitrogen balances throughout the period of observation.

#### DISCUSSION OF BALANCES

Summaries of the balances of all elements followed in the three subjects have been recorded in tabular form. Table I shows the balances of the three men during the control period, Table II the balances during the period when potassium citrate was administered, and Table III the balances during the administration of calcium chloride. In order to present some of the data graphically, two charts have been prepared. Chart 2 shows the changes taking place in the metabolism of calcium, magnesium and phosphorus in each subject during the three periods; similarly Chart 3 shows the changes occurring in the metabolism of sodium, potassium and chlorine.

*Calcium, Magnesium and Phosphorus Balances. Control Period.* An appreciation of the level of intake, mode of excretion, and balances of these elements may best be obtained by reference to Chart 2.

It will be noted that the phosphorus balances are slightly positive in both normal subjects and slightly negative in the nephritic subject during this period. The calcium balance is almost zero in the first normal subject, slightly positive in the second, and slightly negative in the nephritic subject.

The exceedingly low calcium excretion by way of the kidney in Bright's disease, particularly that type in which the tubular lesion predominates,

### CALCIUM, MAGNESIUM, AND PHOSPHORUS BALANCES

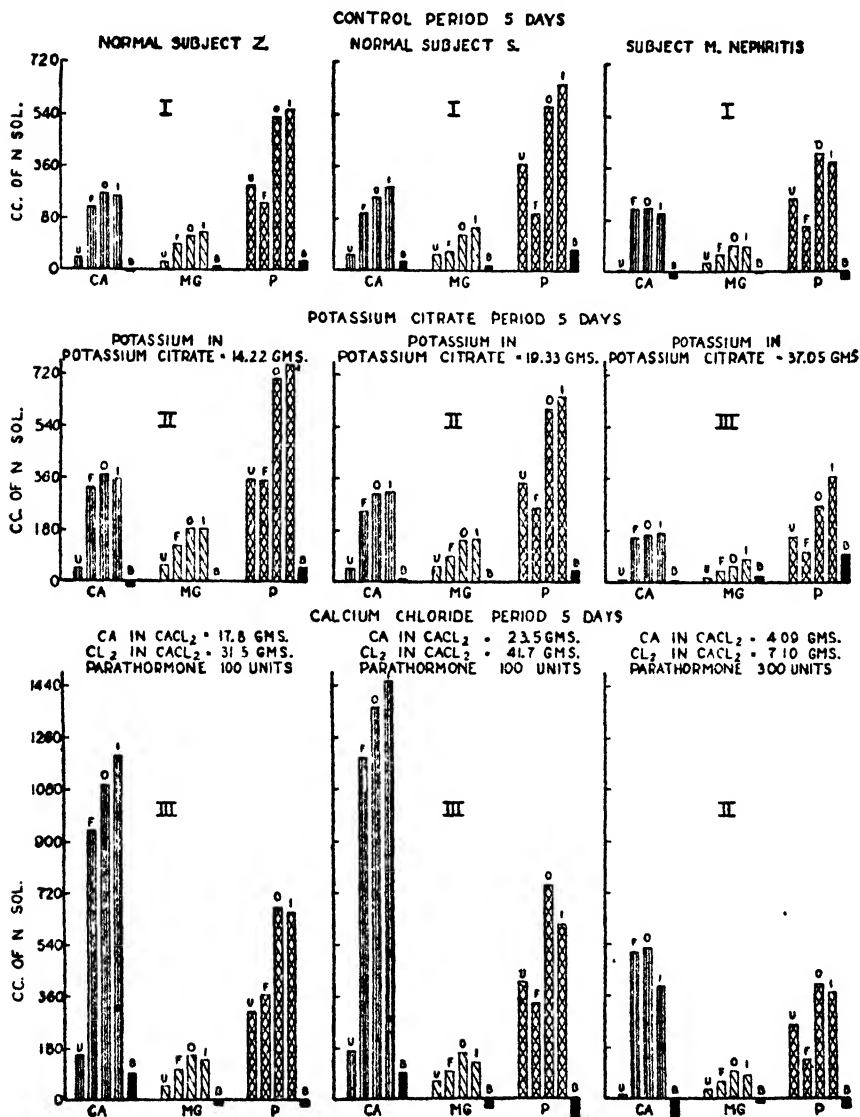


CHART 2. Columns represent cc. of normal solution per 5 day period. Legend. *U* = output in urine; *F* = output in feces; *O* = total output (*U* + *F*); *I* = total intake (food and medication); *B* = balance, positive above base line, negative below base line. Heavy black line in *Ca* intake column separates food intake (below) from medication intake (above). Roman numerals indicate chronological order of periods.

has been discussed by Scriver (30), Albright and Bauer (2) and others. In the nephritic patient it amounted to only about 30 mgs. in 5 days. The partition of magnesium and phosphorus between urine and stool in this man, on the other hand, was essentially normal.

*Potassium Citrate Feeding.* The effect of feeding extra potassium in the form of potassium citrate does not appear to have influenced materially the balances of calcium and phosphorus in the two normal subjects. The positive magnesium balances noted during the previous period in these subjects are no longer present. This perhaps indicates too short a control period on the standard diets rather than an effect due to potassium.

The changes in the balances of calcium and magnesium noted in the man with nephritis were hardly sufficiently striking to be attributable to potassium. The positive balances in his case may well represent compensatory retention following a previous excessive excretion of these elements, since the period of high potassium feeding in this instance followed directly after the one in which calcium chloride and parathormone were administered. Likewise, his positive phosphorus balance may be compensatory for losses of this element during the two preceding periods.

*Citrate Effect.* In the preceding discussion it has been assumed that the citrate radical would be almost completely oxidized in the body and that a consideration of its possible effect on mineral metabolism might therefore be disregarded. Recent studies by Proverman and Brull (26) seem to show that the citrate radical when ingested in sufficiently large amounts (1 gm. of the sodium salt per kg. of body weight) causes an increased elimination of calcium in the urine of dogs. Translated in terms of human dosage this would mean the administration of between 60 and 70 gm. of sodium citrate per diem to a man of average weight. Since our nephritic patient showed minor toxic symptoms while taking 20.5 gm. of potassium citrate a day, it is doubtful whether such a large dose would be tolerated by the human subject. Smaller doses of potassium citrate, as taken by the subjects of this experiment, did not produce an increased excretion of calcium in the urine.

*Calcium Chloride Feeding.* The results obtained in the normal subjects in this period are hardly comparable with those obtained in the patient with nephritis. The doses of calcium chloride given the normal men were enormously greater than those given the nephritic subject. Still further confusion resulted from the fact that parathormone was administered to all three individuals. The normal subjects received but one dose of 100 units of parathormone near the end of this experimental period, while the man with nephritis received a total of 300 units during the course of 5 days.

**Calcium.** From the observations of Scriver (30) one might be led to discount the effect of parathormone in the particular type of Bright's disease exhibited by the nephritic patient. Two observations made are in accord with those of Scriver: a.—the blood calcium was not raised above the normal level following the administration of parathormone as is usually the case in normal individuals, b.—there appeared to be an excessive fecal calcium excretion leading to a distinctly negative calcium balance. Whether the excessive excretion was due to the effect of parathormone, to lack of absorption of ingested calcium, or to the relative inactivity of the subject, it is impossible to determine. Greenwald and Gross (16) found that the administration of parathyroid extract to dogs for short periods of time increased the fecal excretion of calcium. Albright, *et. al.* (3), on the other hand, concluded that parathormone was without effect on the fecal excretion of calcium in man. This subject, together with observations on the effect of activity on fecal calcium excretion, has been fully reviewed by Bulger, Dixon, and Barr (10).

The urinary calcium excretion was also increased, either due to parathormone or to the administration of an acid-forming calcium salt. Judging from observations made by Albright and Bauer (2) on the effect of ammonium chloride on calcium excretion in "nephritis," the latter factor would appear to be the important one. In the normal subjects, high calcium feeding led to a definite retention of calcium during this period. That this retention may be more apparent than real cannot be denied, since the period of high calcium feeding was not followed by a control period on standard diets. As might be expected, owing to the ingestion of an acid-forming calcium salt, the urinary calcium excretion was greatly increased; nevertheless, the major portion of the ingested calcium appeared in the feces.

**Magnesium.** The negative magnesium balances in all three subjects are probably due to the acid effect of calcium chloride, since all show a somewhat higher urinary magnesium excretion than during the control period. On the other hand, evidence concerning the effect of parathormone (16, 34) and of the calcium ion (21, 8) on magnesium metabolism is still inconclusive.

**Phosphorus.** Briggs (9) has called attention to the effect of calcium acetate and calcium chloride on the partition of phosphorus between urine and feces. Feeding large amounts of these salts increased the amount of phosphorus in the feces and diminished it in the urine. The total excretion of phosphorus was diminished. Bulger, Dixon and Barr (10) in discussing phosphorus metabolism in hyperparathyroidism have noted

the essential independence of the absorption of calcium and phosphorus from the intestine in that disease and suggest that their observations are in accord with the studies of Bergeim (6) on the absorption of these two elements. There seems to have been a tendency for the normal subjects in our balance experiments to excrete a somewhat greater proportion of the phosphorus in the stool during the period of high calcium intake than during the control period. The difference in the mode of excretion was not marked however and there was no great preponderance of fecal over urinary excretion in either man. The phosphorus balances were negative. Subject Z, who had a markedly positive nitrogen balance (Table III) during this period, had only a very small negative phosphorus balance, while in Subject S, who was in nitrogen equilibrium, the phosphorus balance was distinctly negative. It is not altogether certain whether these negative phosphorus balances were due to the large doses of calcium chloride (28) ingested or to the effect of parathormone on phosphorus metabolism recently stressed by Albright, *et al.* (3). In summing up the situation three factors must be kept in mind, a.—the effect of the calcium ion tending to increase the excretion of phosphorus in the feces, b.—the effect of parathormone tending to increase urinary phosphorus excretion and c.—the acid effect of calcium chloride which, according to the observations of Fitz *et al.* (12) and Albright and Bauer (2), might also be presumed to cause an increased urinary excretion of phosphorus.

The manner and amount of phosphorus excretion in the man with nephritis varied but little from that of the control period although the balance was negative. It seems probable that neither calcium chloride in the amount given, nor parathormone, influenced the phosphorus metabolism in his case.

#### SODIUM, POTASSIUM AND CHLORINE BALANCES

It is at once apparent from a comparison of Charts 2 and 3, that the manner of excretion of these elements differs fundamentally from that of calcium, magnesium and phosphorus. Sodium and potassium, as is well known, are absorbed almost completely from the gastro-intestinal tract and excreted in the urine in combination with various acid radicals. The amount of sodium excreted in the stool is negligible in all our cases; the amount of potassium excreted by this route, while small, is fairly constant and presumably comes from undigested vegetable residue. The chlorine intake is based upon calculations made from tables (29) and the output does not include chlorine lost in the stool.

*Control Period.* During the control period, one normal subject lost both

sodium and chlorine while the other showed positive balances for both elements. Potassium was retained in each instance. In the case of the patient with nephritis, perhaps due to previous restriction of sodium

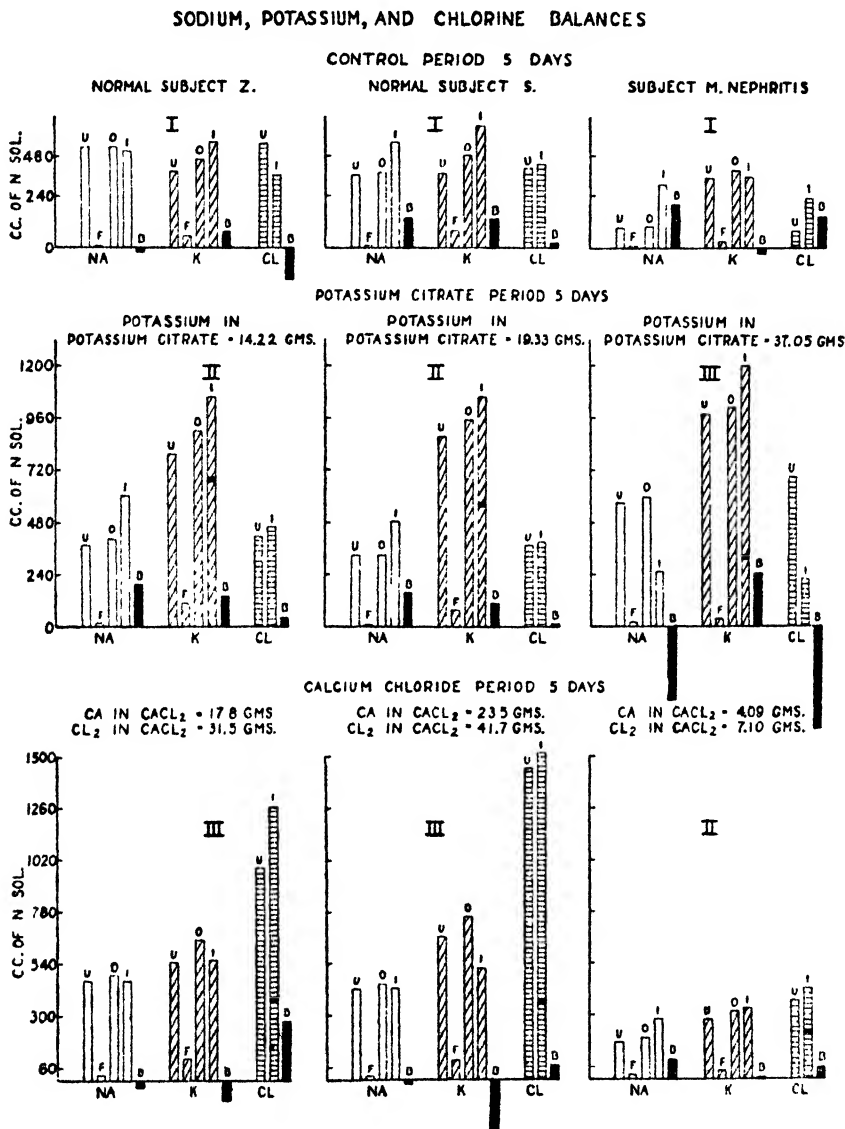


CHART 3. Columns represent cc. of normal solution per 5-day period. Legend. *U*=output in urine; *F*=output in feces; *O*=total output (*U*+*F*); *I*=total intake (Food and medication); *B*=balance, positive above base line, negative below base line. Heavy black line in K and Cl intake column separates food intake (below) from medication intake (above). Roman numerals indicate chronological order of periods.



chloride or to a tendency on the part of the body to retain the elements of this salt, a large proportion of the ingested sodium and chlorine was retained. A similar retention of potassium in his case did not occur. The actual balance of potassium was negative, but not sufficiently so to be considered significant. It is interesting to note (Table I and Charts 2 and 3) that sodium and calcium were excreted by the damaged kidneys of this man in relatively very small amounts when compared with their renal excretion in the normal subjects. Potassium and magnesium, on the other hand, were excreted in his urine as efficiently as they were in the urine of the normal men.

*Potassium Citrate Feeding.* The sodium balance in the two normal subjects was positive during the period of increased potassium intake. The chlorine balances were also slightly positive but did not correspond with the retention of sodium. The major portion of the potassium was excreted in the urine although the balances remained positive in both instances. When one compares the dose of potassium given these men—3 to 4 gm. per diem in addition to that contained in their food, with that taken by Bunge (11) who ingested more than 12 gm. in a single day, it seems probable that the dose given our normal subjects was too small to produce negative sodium balances.

A pronounced effect was noted in the case of the oedematous patient who received 7 gm. of extra potassium per diem. The increased excretion of sodium and chlorine in the urine was striking and resulted in marked negative balances for both elements. (Table II and Chart 3.)

Sweating, diuresis and weight loss accompanied the increased excretion of sodium chloride (See Chart 4). The decrease in volume of total body fluid indicated by weight loss must have been accomplished chiefly at the expense of extracellular water since sodium was the only base materially affected.

*Calcium Chloride Feeding.* The criticisms relative to salt dosage and parathormone administration made in discussing the calcium, magnesium, and phosphorus balances in this period, are applicable to the balances of sodium, potassium and chlorine.

The sodium balances in both normal subjects were slightly negative, but only a very small amount of base was withdrawn from the body in this way. The potassium balances were distinctly negative. Some of the chlorine derived from calcium chloride may have been excreted in combination with potassium, or the negative balances may represent a delay in excretion of potassium, since a control period did not intervene between the periods of high potassium and high calcium intake.

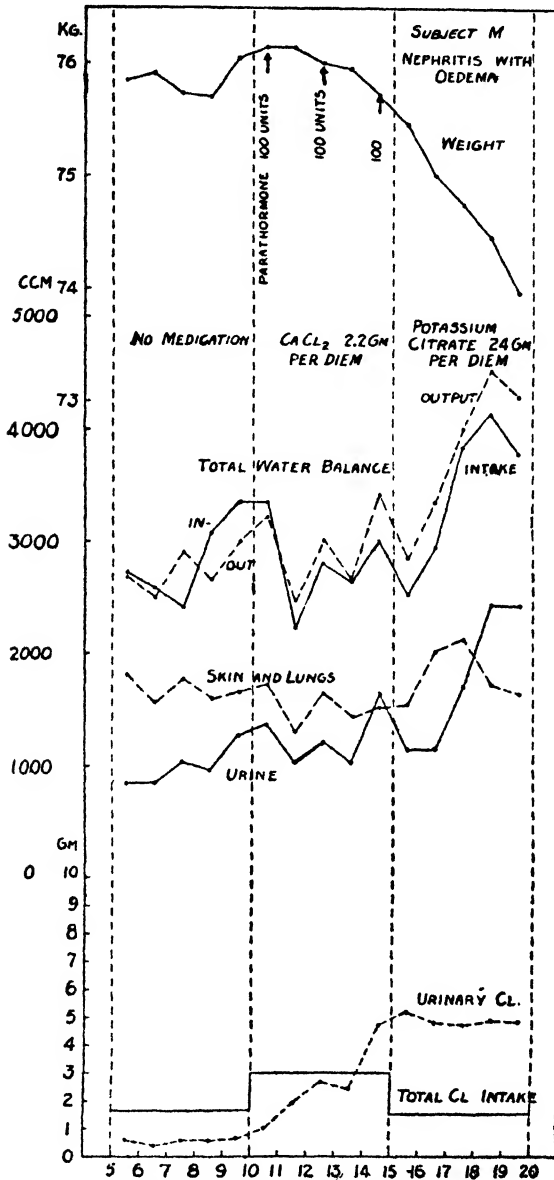


CHART 4. Water balance of Nephritic Subject during mineral metabolism study. Note diuresis, weight loss and increased excretion of chlorine in urine at time potassium citrate was administered.

The chlorine balances were positive throughout but were small except in the case of normal subject Z. The rather large positive chlorine balance in his case may in part have been compensatory for chlorine lost during the control period.

## WATER BALANCES

The method used in calculation of the water balance has been described in a previous publication (4). In the case of the nephritic subject this was determined with sufficient care to permit graphic representation (Chart 4). During the control period he showed a tendency to retain water which led to an increase in weight. The maximum weight for the period of observation was attained during the first two days of calcium chloride ingestion and thereafter fell steadily. The increase in weight corresponded roughly with the retention of sodium and chlorine. The most rapid loss of weight occurred during the period of potassium citrate administration, and, as previously stated, was accompanied by a very definite loss of sodium and chlorine from the body. The excretion of chlorine was in excess of the excretion of sodium and as the daily urinary chlorine curve shows, this was probably due to a lag in the chlorine excretion which occurred at the time calcium chloride was administered.

Owing to the fact that a control period did not intervene between the period of calcium chloride parathormone administration and the period of potassium citrate feeding, the question might well be raised as to whether the weight loss in the latter period actually resulted from the ingestion of potassium or whether it merely represented the continuation of a process initiated by the previous medication. In respect to this point it is significant that the patient lost 5.5 kg. in weight on a previous occasion when potassium citrate was administered in doses of 20 gm. per diem for eight days, (Chart 5).

Coincident with the weight loss there was a moderate increase in urine volume and a three-fold increase in the urinary chlorine excretion. Diet, water intake and sodium chloride intake had been maintained at a constant level for a period of twelve days prior to the beginning of the experiment, without significant change in the patient's weight, and these factors were not varied during the experimental period.

So far as could be determined from weight observations alone, the water balances of the normal men were not materially affected by the smaller doses of potassium citrate which they received. Calcium chloride feeding, on the other hand, caused both subjects to lose weight. Subject Z who had a strongly positive nitrogen balance during this period, lost 0.9 kg. in weight, while subject S who was in nitrogen equilibrium lost 1.8 kg.

## NITROGEN BALANCES

The protein content and caloric value of the diets given the normal subjects approximated the standards given by Atwater (cited by Sherman

(32)) for normal men engaged at moderately active work. Both men showed positive nitrogen balances during part of the period of observation. In the control period, subject Z complained of hunger on a diet whose

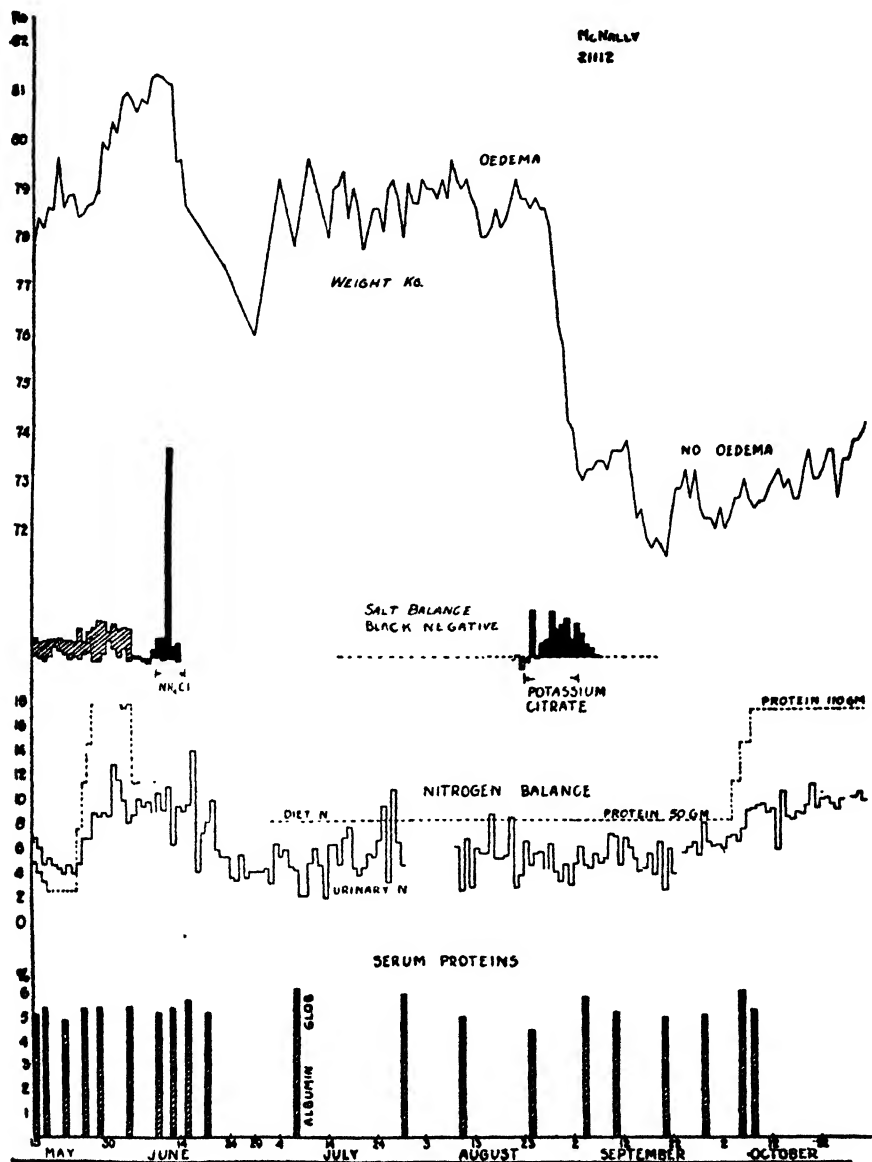


CHART 5. Loss of weight and negative salt balance (sodium chloride as calculated from chlorine excreted in urine) obtained by administration of 20 gm. of potassium citrate per diem to the nephritic subject. Mineral balance not kept at this time.

energy value was about 3000 calories per diem and showed a small negative nitrogen balance. In the two following periods, when the diets had been increased to an energy value of about 3500 calories per diem, he stored approximately 30 gm. of nitrogen.

Subject S, on the other hand, received 3500 calories from the beginning of the experiment. He showed a positive nitrogen balance of 23 gm. during the first ten days and in the final 5-day period seemed to have attained nitrogen equilibrium with his diet. The storage of nitrogen in these two students may indicate a somewhat less liberal allowance of protein in their customary diets than is usually considered desirable.

The nephritic subject, because of his restricted activity, required only about 2000 calories per diem to meet his energy requirements. He maintained a fairly constant weight except when loss occurred during the period of potassium citrate feeding. Even during this period of rapid weight loss he stored nitrogen. It seems reasonable to suppose that nitrogen retention in his case followed as a logical result of the previous restriction of diet protein employed as a therapeutic measure.

#### IRON

The quantity of food iron required for the maintenance of equilibrium in normal men has been discussed at some length by Sherman (31, 32), who has pointed out the rather serious deficiency of experimental evidence in support of commonly accepted standards. Recent investigations carried out by Lintzel (20) would indicate that in healthy adults the excretion of iron is almost exclusively in the feces and that the amount excreted is approximately equal to the amount ingested. He found that balance was maintained on intakes ranging from 0.9 to 64 mg. per diem. Increasing the iron intake from a normal value of 10 to 13 mgs. to 50 or 60 mgs. a day by addition of ferric chloride to the diet, did not result in any appreciable retention of iron. The data given in Lintzel's experiments are rather convincing but in respect to maintenance of iron balance on a practically iron-free diet, his results await confirmation by other investigators.

The iron balances of the three subjects in our experiment were determined with a view to establishing, if possible, the adequacy or inadequacy of the iron content of the diets used. The normal subjects received an amount of food iron averaging slightly in excess of 21 mg. per diem during the entire period of observation. This was considerably above the standard requirement of 15 mg. per diem suggested by Sherman (32) and probably exceeded the usual daily iron intake of the subjects. Both

men stored iron throughout the experiment. Subject Z retained 87 mg. and subject S, 77 mg., a daily positive balance of over 5 mg. in each case. It is possible that this comparatively large storage of iron in our normal subjects may have been associated with some previous impoverishment of their iron reserves, or perhaps simply represented the building up of a reserve iron supply. The latter supposition is more in accord with experimental evidence (18, 36).

The patient with nephritis received an average daily iron intake from his food of 12.2 mg. During the control period he excreted a very large amount of iron in the stool resulting in a negative balance of 309 mg. Whether this represented a contamination of the stool specimen with iron, or a delayed excretion of medicinal iron given prior to the metabolism study, is not known. That it did not represent a loss of body iron seems fairly certain since in the two remaining periods he remained practically in equilibrium with the iron in his diet.

In only one instance did the daily urinary excretion of iron exceed more than a fraction of a milligram, and this occurred in the nephritic subject during the period of high potassium feeding. Even here the amount excreted (6.7 mg. in 5 days) was too small materially to effect the balance.

It should be emphasized that the figures given for iron excreted in the urine are only approximate. The quantity present was found in most instances to be too small for accurate determination. It is <sup>becom. &</sup> <sub>v. and</sub> that the amount of iron in urine has been overestimated rather than underestimated.

## SUMMARY

### *1. Calcium, Magnesium and Phosphorus Metabolism.*

a.—Potassium citrate when added in quantities of 7.8, 10.5 and 24.0 gm. respectively to the diets of two normal men and to the diet of a patient with nephritis and oedema, for periods of five days in each instance, did not alter the metabolism of calcium, magnesium or phosphorus.

b.—The ingestion of 9.8 and 13.0 gm. of calcium chloride per diem by the normal men for five-day periods led to marked increase in the urinary and fecal calcium excretion and at least temporarily to positive calcium balances. In the nephritic subject where the ingestion of 2.2 gm. per diem of calcium chloride was accompanied by intramuscular injection of large doses of parathormone, urinary calcium excretion was increased but the quantity excreted was much less than that excreted by the normal men during the control period. Fecal calcium in his case was excessive and resulted in a distinctly negative calcium balance.

c.—The magnesium balances of all three subjects became negative during the period of calcium chloride—parathormone administration.

d.—The influence of calcium chloride feeding on phosphorus metabolism could not be completely evaluated because of the number of variable factors entering into the experiment.

## 2. Sodium, Potassium and Chlorine Metabolism.

a.—High potassium feeding for five-day periods did not produce negative balances in the metabolism of sodium and chlorine in the normal subjects.

b.—The much larger doses of potassium citrate taken by the nephritic subject caused a marked increase in the excretion of sodium and chlorine in his urine, which resulted in negative balances for both elements. Diuresis, sweating and weight loss accompanied the increased excretion of sodium and chlorine.

c.—In the normal subjects, sodium and potassium balances were negative during the period of calcium chloride feeding. This is believed to have been due to the acid effect of calcium chloride but the negative potassium balances may have been due to delay in excretion of this element resulting from administration of potassium citrate in the immediately preceding period.

d.—The comparatively small doses of calcium chloride given the man with edema did not affect his sodium or potassium metabolism.

3. A question has been called to the unusually small amount of sodium and calcium excreted in the urine of the nephritic subject during the control period, and this has been contrasted with his normal urinary excretion of potassium and magnesium.

4. The total nitrogen metabolism was not affected by the administration of potassium citrate or calcium chloride.

5. Iron was stored by both normal subjects on an average food iron intake of 21 mg. per diem. In the nephritic subject, if one excludes the control period, a food iron intake of 12 mg. per diem was sufficient to maintain iron balance.

## CASE REPORT

### SUBJECT M.

UNIT No. 21112

*History of Illness:* A clerk, aged 44 years, was admitted to the Medical Service of the Strong Memorial Hospital on January 20, 1929, complaining of shortness of breath, palpitation of the heart and swelling of the ankles. His general health had been good until one year prior to admission when he began to suffer from generalized headaches on arising in the morning. These were followed shortly by palpitation of the heart and for the 3 or 4 months immediately preceding admission by shortness of breath. For the past month he had noted considerable swelling of his ankles and had to arise once nightly to urinate.

*Past History* revealed no evidence of illness or disease which appeared to have any relationship to the condition for which he was admitted.

*Family History:* Father died age 53 years, supposedly with Bright's disease.

*Physical Examination:* Temperature 37°C. Pulse 72. Resp. 20. Ht. 169 cm. Wt. 88 kg. The patient was well developed with slight tendency to obesity. There was no orthopnea, dyspnea or cyanosis while at rest. The gums showed moderate pyorrhea and several of the teeth were carious. The pharynx was moderately injected and the tonsils were embedded and reddened. A small amount of pus could be expressed from the crypts of the right tonsil. There were a few moist rales at the lung bases posteriorly. The heart was enlarged to the left, the outer border of cardiac dullness measuring 14 cm. to the left of the mid sternal line in the 5th intercostal space. There was a soft systolic murmur at the apex. Peripheral and retinal arteries showed only a very slight degree of sclerosis. The blood pressure was 160 mm. Hg. systolic and 110 diastolic. The abdomen was soft. The liver and spleen were not palpable. There was moderate pitting oedema over both tibiae.

*Laboratory Examinations:* Urine—specific gravity 1020. Albumin 4 plus, sugar and acetone negative. Microscopic—a few red blood cells but definitely more than normal, and a few granular casts. Stool examination was normal. Blood—Hb. 100 per cent (Sahli); white blood cells 7,000 with a normal differential leucocyte count; N.P.N. 33 mg. per cent; serum albumin 4.3 per cent globulin 1.6 per cent; blood Wassermann negative. Electrocardiogram showed left ventricular preponderance. X-rays of the chest showed definite cardiac hypertrophy. An intravenous phenol-sulphonphthalein test showed 60 per cent excretion of the dye in the urine in two hours. X-rays of the teeth showed some alveoloclasia and pyorrhea.

*Hospital Admissions: First Admission,* January 20, 1929 to February 17, 1929. The patient remained in bed throughout this period of approximately four weeks. He was placed on a diet of low sodium chloride content with some decrease in the oedema of the lower extremities but this did not entirely disappear. There was a gradual fall in the blood pressure to 132 systolic and 84 diastolic. The rales heard at the lung bases on admission soon cleared and there remained no signs of cardiac decompensation. The urine continued to show a large amount of protein, generally about 6 to 8 gm. per liter but at times as high as 20 gm. per liter. Red blood cells and granular casts were consistently found in the urine. On February 5, 1929 the tonsils were removed, and a small abscess was found in the right tonsil. Although there was no excessive bleeding as the result of the operation, the patient suffered somewhat from shock and recovered slowly. Examination of the blood just prior to discharge showed essentially the same findings as on admission. He was discharged with the advice to rigidly curtail his activity and to have all remaining infected teeth extracted. Dietary restrictions other than keeping sodium chloride intake at a low level were not imposed.

*Second Admission:* May 14, 1929 to December 21, 1929. The patient was admitted the second time primarily for study of his nitrogen and mineral metabolism. The physical and laboratory examinations were essentially as given for the first admission. During the first two weeks the effect of low and high protein intake on the course of oedema was observed while the patient was on a sodium chloride intake of about 3.5 gms. per diem, and later a low sodium chloride intake (1.5 gms. per diem) was given with a protein intake of about 20 gm. a day. Weight tended to increase during most of the month of observation with perhaps slight increase in the oedema. Blood N.P.N., serum albumin, globulin and total serum protein showed some variation but probably not significant amounts. Average values were N.P.N. 30 mg. per cent, serum albumin 4.5 per cent, serum globulin 0.8 per cent and total serum protein 5.3 per cent. On June 16, 1929 the patient developed bronchopneumonia from which he recovered very slowly. Oedema of the legs became pronounced during the period of recovery with more marked swelling of the right leg than the left suggesting a low grade phlebitis. Metabolism studies had to be discontinued indefinitely. During July and August the urine repeatedly showed many hyalin, granular and cellular casts. The blood



showed moderate secondary anemia, red blood cells, 4.2 millions, Hb. 66 per cent. From August 26th to September 3rd he was given daily doses of 20 gm. of potassium citrate with a reduction in weight from 78.6 to 73.2 kg. and improvement in oedema. Toward the end of October his weight gradually increased again and the oedema of the lower extremities became more marked. Urea clearance test (Van Slyke) October 22, 1929, showed a maximum clearance of about 47 per cent of the normal average. On December 5, 1929 he was readmitted to the metabolism unit for a period of 15 days during which a study of mineral exchanges was carried out as reported in this paper.

At the time of discharge December 21, 1929, the blood count showed red blood cells 5.4 millions, Hb. 82 per cent, white blood cells 10,000. Blood serum protein 4.78 per cent. Urea clearance test (Van Slyke), standard clearance 48 per cent of normal average. Urine, concentrated night specimen 32,000,000 red blood cells for 12 hours (Addis count) very few casts or white blood cells. Protein 4.4 gm. per 12 hours. Specific gravity 1018.

*Clinical Impression:* Nephritis, subacute hemorrhagic; Hypertension; Heart disease, hypertensive; Bronchopneumonia; ? Phlebitis, right leg.

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# EFFECT OF VITAMIN WITHDRAWAL ON THE MONKEY (MACACUS RHESUS)

By

R. G. TURNER AND E. R. LOEW

*(From the Medical Research Department, Detroit College of  
Medicine and Surgery, Detroit)*

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**E**XPERIMENTAL and clinical evidence has clearly established that deficiency of vitamin A produces a pathologic entity classified as xerophthalmia. The lowered resistance to infection and the development of lesions appear to be secondary to the development of xerophthalmia. These lesions occur in the cornea, nasal passages, accessory sinuses, middle ear, tongue, and upper respiratory tract.

The experimental production of this condition in eyes and suppurations in the upper respiratory tract of the rat do not prove that similar symptoms will appear in different species of animals under like experimental conditions.

We look to biochemistry to explain many of the mysteries that surround us in the various epidemics of influenza, tonsillitis, mastoiditis or diphtheria. Why does acute sinusitis, tonsillitis, acute laryngitis or mastoiditis prevail in epidemic form and change in type overnight to a greater or lesser degree of virulence and become engrafted on a new area of mucous membrane or lymphoid tissue? If the influence is climatic or microbic, the host with lowered resistance for the particular type of infection succumbs. An understanding of the true factors that determine this resistance may be dependent on a changing biochemistry of the cell with a changing virulence of the micro-organism. From the study of infections in the upper respiratory tract in the rat and monkey this theory has been more clearly substantiated.

This phenomenon, described as xerophthalmia, a disease of the eyes, has been observed by McCollum (1), Drummond (2), and other investigators in the albino rat. Ophthalmia has been observed in dogs (3), rabbits (4), calves (5) and chickens (6). Wolbach and Howe (7) were unable to produce the disease in guinea-pigs.

Evans and Bishop (8) in 1922 observed a change in the epithelial cells of the vaginal wall in rats suffering from lack of vitamin A. Goldblatt and Benischek (9) later showed that this metaplasia likewise was encountered in the tissues of the upper digestive tract and nasal passages. In 1923

Daniels (10) reported that rats afflicted with xerophthalmia often showed suppurations in the nasal cavities and sinuses. This characteristic susceptibility of the rat developing from withdrawal of vitamin A has been confirmed by Macy (11), Sherman (12), Mellanby (13), and Turner (14).

The experimental production of xerophthalmia and infections of the upper respiratory tract in the rodent do not prove that similar symptoms will appear in all species of animals under like experimental conditions. It was thought that similar studies on the monkey might aid in making more clear the relationship of diet to upper respiratory and sinus infection in the human.

Reports on symptoms of the eye disease in monkeys placed on artificial diets free from fat-soluble vitamin A, are rare. McCarrison (15) 1920, placed monkeys on a ration which lacked vitamins A and C. He did not observe an ophthalmia in any of his monkeys but regarded the gastrointestinal disturbance which developed as due to the absence of these vitamins.

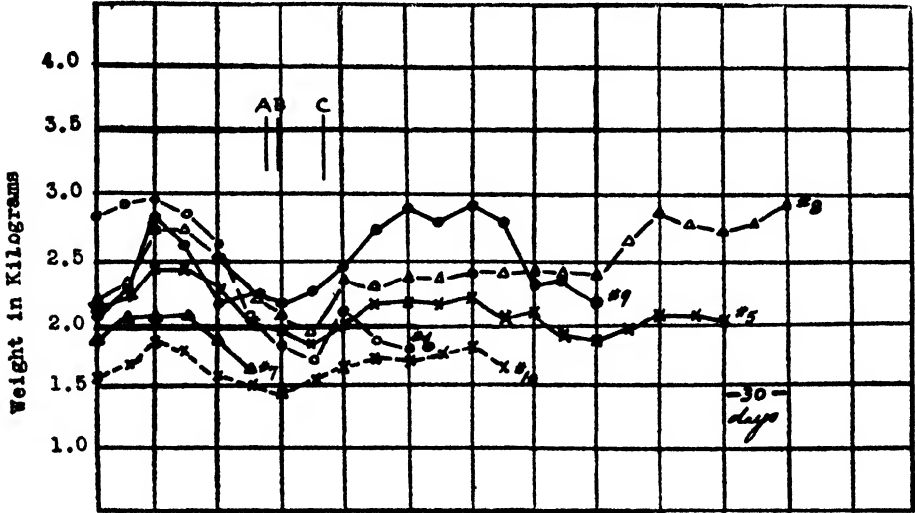
Saiki (16) 1929, studied the liver function of monkeys in which a true fat-soluble deficiency disease was produced. He reports on two animals which showed symptoms of xerophthalmia on the 30th and 47th days of feeding a diet free from vitamin A.

Tilden and Miller (17) 1930, have recently reported observations on 11 monkeys placed on a ration containing 6 to 12 units of vitamin A daily. In no instance did they encounter eye lesions, suppurations in the nasal cavities or in the middle ear. They observed keratinization of epithelial tissue in one or more sites in 9 of the 11 animals. Colitis and gastro-intestinal disorders were the chief lesions observed at autopsy.

The experiments carried out in the laboratories of the Detroit College of Medicine and Surgery include a study on two groups of monkeys. Observations were made on six monkeys (2 to 4 years of age) placed on a diet deficient in vitamins A and C (McCarrison's) followed by a diet deficient in vitamin A (Saiki's) until death. Vaginal smears were taken three times a week and the results recorded with the menstrual dates. The second group consisted of eight monkeys (1 to 2 years of age). These monkeys received the Saiki diet only. In this group a histological study was made of tissues from the bladder, spleen, intestines, trachea, thyroid, salivary glands, lungs and eyelids. The sections were taken immediately after the animals succumbed. Observations were also made on eight control monkeys (4 with each group) which received diets with adequate amounts of vitamins. Vitamin E was not supplied in any of the diets.

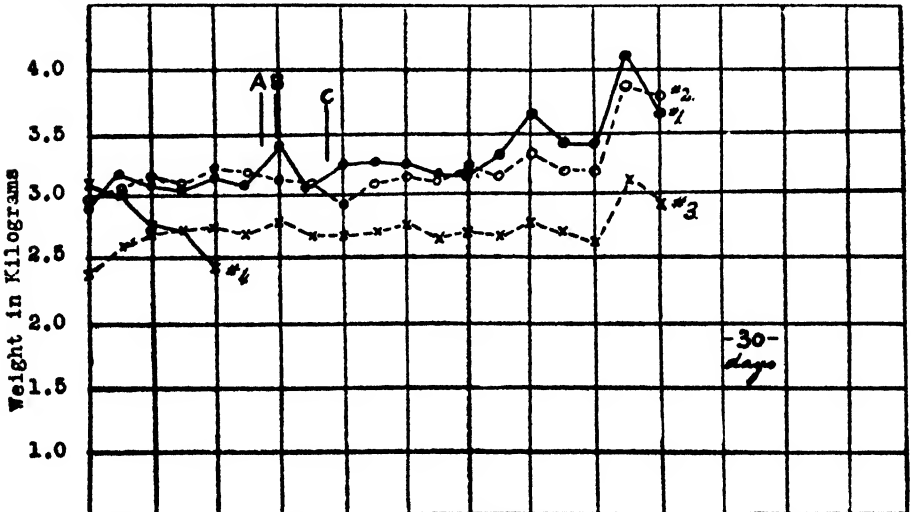
The results of these experiments show that monkeys on a diet deficient

CHART NO. 1. GROWTH CURVES OF MONKEYS PLACED ON VITAMIN-A-DEFICIENT DIET TEST.



- A.—Animals showed weakness and paresis of rear limbs. Purified orange juice added to diet to provide vitamin C.
- B.—Mature females showed continued presence of cornified cells in vaginal smears. Menstruation ceased or interval was greatly prolonged.
- C.—Changed from McCarrison's diet to diet formulated by Saiki.

CHART NO. 2. GROWTH CURVES OF MONKEYS PLACED ON COMPLETE RATION CONTROLS.



- A.—No loss in weight, weakness or paresis of rear limbs. Oranges were included in complete ration from beginning of experiment.
- B.—Mature females did not show continued presence of cornified cells in vaginal smears. Menstruation continued regularly.
- C.—Changed from McCarrison's diet to diet formulated by Saiki.

in both vitamins A and C, develop a scurvy-like condition, indicated within 70 to 80 days by paralysis of the rear limbs, with marked loss in weight. Addition of orange juice (freed from vitamin A) relieved this condition, the animals improving in health and gaining in weight.

No signs of xerophthalmia were observed during the depression. It was thought that the bread or milk used in the McCarrison diet might contain small traces of vitamin A, sufficient to prevent the animal from developing the eye disease. The animals were placed on the Saiki diet on the 110th day. They lived on this diet from 1 to 10 months, finally succumbing with loss of weight and gastro-intestinal symptoms. Abscesses of the tongue

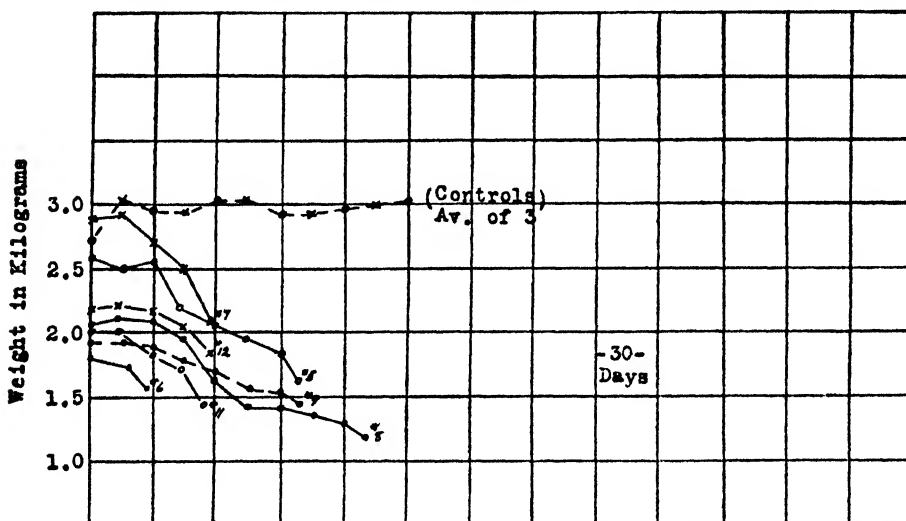


CHART 3. Control animals received 3% C. L. O. in their diet. Curves 5 to 12 inclusive represent growth curves of young monkeys on Saiki Vitamin A deficient diet.

were encountered in one of these animals, while another showed abscesses of the lower molars.

Vaginal smear records showed no consistent variation in the presence of epithelial, leukocyte and cornified cells during the normal menstrual cycle. In mature monkeys the presence of cornified cells was found to persist after 60 days on the vitamin-deficient diet with a complete cessation of the menstrual period. Control mature monkeys showed no abnormalities in the menstrual period and at no time showed the continued presence of desquamated cells.

The younger monkeys lived from 3 to 5 months on the vitamin A-deficient diet, succumbing with symptoms identical to those found in the older monkeys. In general, death of these monkeys on vitamin A-deficient ra-

tions was preceded by loss in weight accompanied with a severe diarrhea terminating in a mucous-like exudate. Postmortem examination showed evidence of intestinal inflammation with marked enteritis and dilatation of

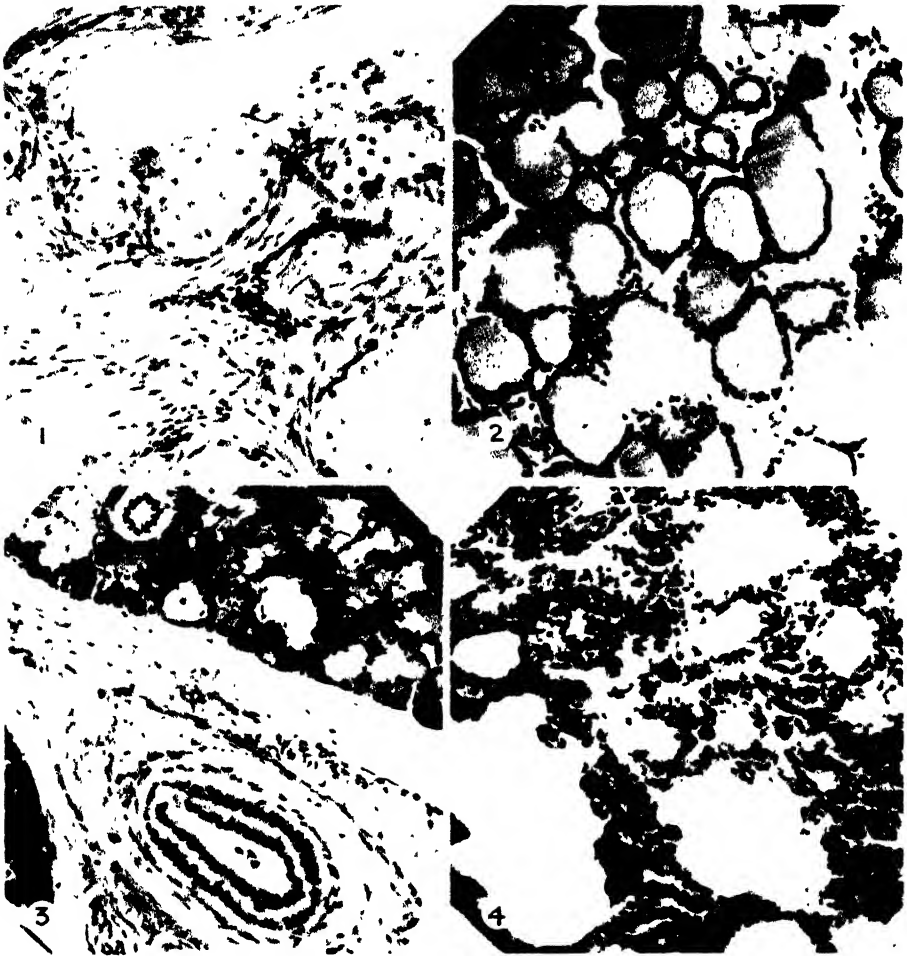


FIGURE 1.

No. 1. Eyelid of monkey No. 8.  $\times 100$ . Showing a follicular process with local aggregation of lymphocytic cells beneath the mucous membrane. No evidence of metaplasia is evident.

No. 2. Thyroid gland of monkey No. 8.  $\times 100$ . Showing no evidence of metaplasia or keratinization of the epithelial cells.

No. 3. Salivary gland of monkey No. 8.  $\times 100$ . Showing no evidence of metaplasia or hyalinization of the epithelial cells.

No. 4. Lung of monkey No. 8.  $\times 100$ . Showing no evidence of bronchial pneumonia or metaplasia of epithelial cells. Atelectasis is evident in a few small areas.



the stomach. Xerophthalmia was not observed. The upper respiratory tract, nasal cavities and middle ear were free from suppuration in all of the deficient animals.

Histological examination of the 8 younger monkeys and monkey No. 8 of the older group, showed no signs of keratinization or hyalinization of the epithelia in the tissue studied.

Most of the control monkeys maintained their weight, with minor fluctuations, throughout the experimental period. Three of the control monkeys died, one showing bronchial pneumonia, the other two symptoms of tuberculosis with abscessed spleen, liver and lungs. The latter picture was not observed in any of the animals succumbing from lack of vitamin A.

In conclusion, it appears that withdrawal of vitamin A from the monkey does not develop a characteristic susceptibility toward infections of the upper respiratory tract. Xerophthalmia, at least, is difficult to produce and no definite change in the tissues of the upper digestive tract are observed.

Thus it seems that infections of the upper respiratory tract brought about by experimental diets are encountered most frequently when that portion of the tract is affected by a change in cell structure.

We wish to express our thanks to Dr. A. L. Amolsch who was kind enough to examine the microscopic tissue preparations and to Dr. Burt R. Shurly whose interest has made this work possible.

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# THE HEMOGLOBIN CONTENT OF THE BLOOD OF DAIRY CATTLE\*

By

H. J. BROOKS AND J. S. HUGHES†

(*From the Kansas Agricultural Experiment Station, Manhattan.*)

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ONE of the problems encountered in studying the efficiency of milk alone as a feed for dairy calves was nutritional anemia. In interpreting results, it was important to know the hemoglobin content of the blood of normal dairy calves. No work could be found reported in the literature on this subject. Dimock and Thompson (1) report an average of 8.96 grams of hemoglobin in 100 cc. of blood in an examination of 20 head of cattle of the four major dairy breeds in the Cornell dairy herd. The maximum was 12.75 grams and the minimum 6.75 grams. However, no determinations were made on calves. In order to secure such information, the hemoglobin content of the blood of the calves in four dairy herds was determined and at the same time a study of all ages was included. Determinations were made in the college herd consisting of the four major breeds of dairy cattle, in the Holstein herd at the Topeka State Hospital, the Holstein herd at the Osawatomie State Hospital, and in a privately owned herd of Guernsey cattle.

In all a total of 335 determinations were made on 297 head.

## METHOD

The acid hematin method (2) was used for the determination of the hemoglobin. A 0.05 cc. sample of blood was taken from the ear vein by means of a capillary pipette and was introduced into 10 cc. of tenth normal hydrochloric acid. After the hemoglobin color had fully developed, the sample was matched against a standard. This standard was made up from blood that had been standardized by means of the Van Slyke method (3) for determining the combined oxygen of the blood. Although perhaps not necessary, a new standard was prepared each week.

The results from the four herds are tabulated in Table I according to the four groups, cows, heifers, calves, bulls. Included in the cow group

\* Contribution No. 76 from the Department of Dairy Husbandry and contribution No. 159 from the Department of Chemistry.

† Acknowledgment is made of the assistance given by Dr. J. L. Hall of the Department of Chemistry in the chemical analyses in this project.

TABLE I  
HEMOGLOBIN CONTENT OF THE BLOOD OF DAIRY CATTLE

Group	No. of animals	Grams of hemoglobin per 100 c.c. blood		Standard deviation
Cows	103	10.94	$\pm .1034$	1.538
Heifers	59	11.71	$\pm .1089$	1.241
Calves	111	10.46	$\pm .1059$	1.654
Bulls	24	11.92	$\pm .2612$	1.893
Average		10.96	$\pm .0638$	1.635
Maximum	3 Yr. Ayr. heifer	16.50		
Minimum	1 Mo. Guer. bull	5.90		

are all females that had calved at least once, in the heifer group, females from 5 to 28 months of age that had not yet calved; in the calf group, animals of both sexes up to 16 months of age that were still maintained with the calf herd, while in the bull classification were included all male animals over 9 months of age. The calf group necessarily included some animals from both the bull and the heifer groups.

As noted in Table I, the hemoglobin content of the blood is rather constant in dairy animals. The individuality of the animal seemed to be the most important factor in causing variations from the normal.

The mean for all determinations was  $10.98 \pm .064$  grams of hemoglobin in the 100 cc. of blood. The group of 24 bulls varying from nine months to fourteen years of age gave the highest reading, followed in close order by the heifer, cow and calf groups. The lowest value of 5.90 grams of hemoglobin in 100 cc. of blood obtained in a single individual was upon a one month old Guernsey bull owned in the private herd. This fact, together with the facts of the low average hemoglobin content of the blood of this Guernsey herd as well as that of the Guernseys in the College herd and the lower average for all Guernseys shown in Table II, suggests a

TABLE II  
SUMMARY OF DATA BY BREEDS

Breed	No. of Animals	Grams Hb in 100 cc. blood	
Jersey	13	11.60	$\pm .2039$
Guernsey	77	10.34	$\pm .1255$
Ayrshire	32	11.44	$\pm .1786$
Holstein	175	11.10	$\pm .0812$

breed difference. However, it is noted that the Holstein herd at the Osawatomie State Hospital showed the lowest hemoglobin content of any herd. It may also be noted in Table III that there is a greater difference between the same group in the respective herds than between groups within a herd. This is rather difficult to explain and suggests further study as to just why such differences between herds should exist.

TABLE III  
SUMMARY OF ALL DATA BY HERDS

Group	College Herd Hol.-Ayr.- Guer.-Jers.		Topeka State Hospital Hol.		Osawatomie State Hospital Hol.		Guernsey Herd	
	No. An.	Hb	No. An.	Hb	No. An.	Hb	No. An.	Hb
Cows	38	11.62	26	11.94	21	10.43	28	9.85
Heifers	19	11.99	30	12.10	10	10.46	—	—
Calves	30	10.38	18	12.68	39	9.46	24	10.13
Bulls	8	12.93	4	12.99	9	10.61	4	10.80
Summary	95	11.36	78	12.27	79	9.97	56	10.05

The maximum value of 16.50 grams of hemoglobin in the 100 cc. of blood obtained in a single individual was found in the case of a three year old Ayrshire heifer in milk in the College herd. In regard to the groups, the Holstein calf herd at the Osawatomie State Hospital consisting of 39 animals gave the minimum value of 9.46 grams of hemoglobin. A group of four Holstein bulls at the Topeka State Hospital averaged 12.99 grams of hemoglobin which was the maximum for the groups. These differences are hardly sufficiently great to be significant.

There seemed to be no significant relationship between the age of the animal and the hemoglobin content of the blood. This fact is brought out in Table IV which shows the summary of the determinations on all cattle grouped in 5-month intervals.

In order to note any variation in the hemoglobin content of the blood from day to day, determinations were made daily on a group of three animals for an eleven day period. These animals showed very little variation throughout the period. The mean for the group for the period was 10.91 grams of hemoglobin, with a standard deviation of 0.416.

Similar results were obtained on a group of five heifers of the four major breeds that were fasted for fourteen days in a blood sugar deter-

TABLE IV  
VARIATION OF HEMOGLOBIN WITH AGE

Age mos.	No. of animals	Hb	Age mos.	No. of animals	Hb
3	54	10.53	73	2	12.23
8	58	10.52	78	6	10.91
13	32	11.21	83	4	11.62
18	14	11.76	88	6	10.08
23	23	11.85	93	8	11.27
28	14	11.73	98	3	10.18
33	12	11.49	103	2	9.59
38	9	11.21	108	2	10.72
43	7	9.98	113	2	11.04
48	6	11.26	118	4	11.36
53	5	10.77	123	2	10.76
58	11	11.18	128	2	11.36
63	4	10.73	133	2	11.95
68	3	11.64			

mination experiment. The mean for this group for the period was 12.98 grams of hemoglobin per 100 cc. of blood, with a standard deviation of 0.721.

### CONCLUSIONS

1. The mean hemoglobin content of the blood of dairy cattle in 335 determinations on 297 head was  $10.96 \pm .064$  grams of hemoglobin in the 100 cc. of blood.

2. Such factors as breed, age, and prolonged fasting did not seem to affect appreciably the hemoglobin content of the blood and no significant individual variation was observed from day to day.

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# FACTORS WHICH DETERMINE THE CONCENTRATION OF CALCIUM AND OF INORGANIC PHOSPHORUS IN THE BLOOD SERUM OF RATS

## *Second Paper*

By

BENJAMIN KRAMER AND JOHN HOWLAND\*

(with the technical assistance of I. F. Gittleman)

(*From The Pediatric Laboratory, Johns Hopkins  
Medical School, Baltimore, Maryland.*)

Received for Publication—April 27, 1931

### INTRODUCTORY REMARKS

IT IS customary to speak of a normal concentration of calcium or of inorganic phosphorus in serum, and if normal individuals subsisting on a well balanced diet are chosen the concentration of these components in serum will be found to be singularly constant (1). Thus, the serum calcium concentration of normal infants free from clinical or x-ray evidence of rickets or of clinical evidence of tetany is found to be  $10 \pm 1$  mg. per 100 cc. of serum, while the inorganic phosphorus is  $5 \pm 0.5$  mg. Measurable deviations from the normal have been found in disease and may be sustained over a long period of time but where such deviations are marked, clinical symptoms soon appear and in some instances are characteristic of the particular inorganic deficiency. A marked decrease in serum calcium has been observed in infantile (2) and parathyroid (3) tetany and in chronic nephritis, particularly the type designated as nephrosclerosis (4). In cases of chronic parenchymatous nephritis with edema and low serum protein there is a parallelism between the reduction in serum protein and in serum calcium (5). A moderate decrease of serum calcium may follow operations on the thyroid gland and has been ascribed to incidental damage to the parathyroids (6). Reductions in serum calcium concentration have been reported in rickets without tetany (7), in osteomalacia (8), in coeliac disease (9), in sprue (10), and recently, in pneumonia (11). Transient increases in serum calcium may be produced in normal individuals or animals by the oral administration of large amounts of soluble calcium salts or the intravenous injection of smaller amounts (12). The repeated injection of parathyroid extract produces a marked hypercalcaemia with vomiting, muscular atony, profound depression, anorexia,

\* Deceased.

ataxia, bloody stools, and other symptoms (13). Hypercalcaemia may follow the administration of toxic doses of irradiated ergosterol (14).

The concentration of inorganic phosphorus in blood serum is not so constant as that of calcium. The low inorganic phosphorus of severe infantile rickets and of the experimental form of this disease, is well known. Gerstenberger and his collaborators have reported low values in pneumonia (15), in the absence of demonstrable rachitic changes in the bones or in spite of anti-rachitic therapy. A marked elevation of the inorganic phosphorus level in serum is a common finding in nephrosclerosis, where it is often accompanied by a proportional decrease of the serum calcium, a reciprocal relationship which has been frequently observed (16).

In 1922 (17), we reported some observations on a series of rats which had been fed on diets that allowed normal growth for at least four generations and a maximum longevity. The value obtained for serum calcium in these animals was  $10 \pm 0.5$  mg. in 100 cc. of serum; for inorganic phosphorus  $7.7 \pm 0.7$  mg. Two basal diets were used, one deficient in calcium and the other in phosphorus. To these basal diets various additions, including cod liver oil and other fish oils, vegetable oils and butter fat, were made. In some experiments, the calcium phosphorus discrepancy was equalized, by the addition of salts of calcium or phosphorus or an organic phosphorus compound. Other groups of animals were exposed to radiations emanating from various sources and containing varying amounts of ultra violet light of wave lengths less than 3000 Ångstrom units.

Our conclusions may be summarized as follows:

The concentration of calcium and of inorganic phosphorus in the serum is determined by at least four factors, namely, the calcium content of the diet, its total phosphorus, the ratio of these two components in the diet and the amount of vitamin D preformed or produced by irradiation. Without the balancing effect of vitamin D in the form of cod liver oil, butter fat, or ultra violet light the calcium and phosphorus concentration of the serum varies directly with the concentration of the same element in the diet. A discrepancy between the concentration of these two elements in the diet is reflected as a similar discrepancy in the blood serum.

In 1923 a contribution by Park and his collaborators (18), appeared bearing on the same point. Rats were fed a basal ration low in calcium, phosphorus and fat soluble organic factor (calcifying factor). A number of diets were prepared by adding different amounts of calcium carbonate or disodium phosphate or both to this basal diet. The experimental diets were fed for 33 to 35 days. At the end of this period the control animals were killed and calcium and inorganic phosphorus determined on the serum. Two per cent cod liver oil was added to the diet of the remaining animals and these were also sacrificed at the end of ten days. The cod

liver oil addition increased the calcium concentration in each instance. With the low calcium, low phosphorus diet the serum calcium was increased but the inorganic phosphorus remained practically unaltered. The marked discrepancy between calcium and phosphorus in the diet was reflected in the concentration of these elements in the blood of the control animals. A low calcium/phosphorus ratio in the diet yielded a similar ratio in the serum and vice versa. This discrepancy was partly equalized after cod liver oil feeding. To quote Dr. E. A. Park:

The experiments demonstrate in a striking manner the regulatory power of cod liver oil on the calcium and phosphorus metabolism of the organism. The changes in the concentration of the calcium and inorganic phosphorus of the blood serum which occurred under the influence of cod liver oil were striking in degree. Further, cod liver oil not only acts as a regulator of the calcium and phosphorus metabolism, but also permits the organism to operate with greatly increased economy. In the presence of calcium or phosphorus starvation, cod liver oil enables the animal to get along as if the calcium or the phosphorus were supplied in sufficient or almost sufficient quantity in the diet. It is necessary to suppose that cod liver oil brings about maximal utilization of the minimal quantities of calcium or phosphorus in the diet. It greatly reduces waste of these elements and, therefore, must bring about maximal absorption from the alimentary tract. Obviously, cod liver oil makes the metabolic processes of the body, in respect to calcium and phosphorus, vastly more efficient.

Drs. Park, Guy and Powers recognized the regulatory power of cod liver oil on calcium and phosphorus metabolism, a power which was exerted by virtue of the more efficient utilization of these elements under the influence of the oil.

In 1924 and 1925, we had the opportunity of analyzing the sera of a large number of rats that were the subject of an experiment by Prof. McCollum and Miss Simmonds, to determine the effect upon bone development, growth, etc., of quantitative variations in the concentration of calcium, inorganic phosphorus and calcifying factor in the diets of rats. Through the courtesy of Prof. McCollum and Miss Simmonds we are reporting these results. Although our knowledge of the process of calcification, of rickets and other bone diseases and of the factors which determine inorganic equilibria in serum has been substantially enhanced since this work was completed, it is believed that the results are of sufficient interest to justify their publications at this late date.

#### EXPERIMENTAL

"The rats were fed a basal diet consisting of the following:<sup>1</sup>

Rolled oats.....	40.0 gm.
Gelatin.....	10.0 "

<sup>1</sup> The experiments were carried out in Prof. McCollum's laboratory in the Department of Chemical Hygiene, of the School of Hygiene, of The Johns Hopkins University. At the end of each experiment the animals were weighed and then sent to us for the analysis of their blood serum.



Wheat gluten.....	7.0 "
NaCl.....	1.0 "
KCl.....	1.0 "
Dextrin.....	41.0 "

This diet contained 0.181 grams of phosphorus and 0.045 of calcium in 100 grams of food. The proportion of calcium to phosphorus was varied by adding  $\text{CaCO}_3$  or  $\text{KH}_2\text{PO}_4$  or both. The diets were fed for approximately six weeks. Vitamin D was fed at different levels by using 1 per cent butter fat, 10 per cent butter fat and 2 per cent cod liver oil. The calcium of the diet was varied from 0.14 to 1.24, the phosphorus from 0.2 to 1.2, grams per 100 grams of food.<sup>1</sup>

At the end of the experimental period the animals were sacrificed, and the sera pooled and analyzed for calcium and inorganic phosphorus. Hemolyzed sera were discarded. Determinations were all done in duplicate. The Kramer-Tisdall method was used for the calcium determinations<sup>2</sup> and the Briggs-Bell-Doisey method for inorganic phosphorus. The animals were weighed at the beginning and at the end of each experiment. The weight gain was recorded for each animal and the average gain for the group was calculated.

#### SERIES 1

In this series of experiments, the butter fat in the diet was maintained at a 1 per cent level. The calcium content was varied from 0.14 to 1.24 per cent and the total phosphorus from 0.2–1.2 per cent. With the lowest calcium concentration in the diet that of the serum remains at the low level of  $5.7 \pm 1$  mg. Lower values seem to be incompatible with life. These animals either lost weight or gained very little.

As the calcium of the diet increased so did that of the serum until at a calcium level of 1.04 per cent in the diet, that of the serum was normal. When phosphorus was fed at a level of 0.8 per cent it was necessary to increase the calcium of the diet to 1.24 per cent to attain a normal concentration of serum calcium. As the calcium concentration in the diet and that in the serum increased, the inorganic phosphorus of the serum decreased. With fixed calcium intake the inorganic phosphorus of the serum rose parallel with that in the diet. Thus, with a calcium of 1.24 and phosphorus of 0.2 per cent the serum phosphorus was 3.0 mg. per 100 cc. of serum. With the same calcium concentration in the diet and 0.8 per cent phosphorus the serum inorganic phosphorus was 8.7 mg. When the phosphorus of the diet was raised to 1.2 that of the serum was 9.7 mg. per cent.

In the presence of inadequate amounts of vitamin D there seemed to be

<sup>1</sup> The samples were allowed to stand over night for complete precipitation of the calcium oxalate.

TABLE I  
EFFECT OF BUTTER FAT 1%, PHOSPHORUS 0.2%, VARIABLE CALCIUM  
OF DIET ON CA AND P OF SERUM

Diet	Ca/P in diet	Serum Ca*	Serum P*	Weight gain gm.
P=0.2 Ca=0.14	0.72	5.4	6.5	21
P=0.2 Ca=0.24	1.22	6.2	5.7	31
P=0.2 Ca=0.64	3.22	9.7	5.6	35
P=0.2 Ca=1.04	5.23	10.2	3.9	25
P=0.2 Ca=1.24	6.22	10.2	3.0	20

\* Concentrations expressed in mg. per 100 cc. of serum.

TABLE II  
EFFECT OF BUTTER FAT 1%, PHOSPHORUS 0.4%, VARIABLE CALCIUM  
OF DIET ON CA AND P OF SERUM

Diet	Ca/P in diet	Serum Ca	Serum P	Weight gain gm.
P=0.4 Ca=0.14	0.36	5.3	11.2	26
P=0.4 Ca=0.24	0.61	5.5	8.8	21
P=0.4 Ca=0.44	1.11	6.5	9.5	37
P=0.4 Ca=0.64	1.61	6.9	8.7	28
P=0.4 Ca=0.84	2.11	10.0	6.0	42
P=0.4 Ca=1.04	2.61	11.0		40
P=0.4 Ca=1.24	3.11	11.0	3.5	25

TABLE III  
EFFECT OF BUTTER FAT 1%, PHOSPHORUS 0.6%, VARIABLE CALCIUM  
OF DIET ON Ca AND P OF SERUM

Diet	Ca/P in diet	Serum Ca	Serum P	Weight gain gm.
P=0.6 Ca=0.14	0.24	4.6	11.3*	14
P=0.6 Ca=0.24	0.40	5.8	10.1*	18
P=0.6 Ca=0.44	0.74	5.6	12.2*	20
P=0.6 Ca=0.64	1.07	7.3	11.6	33
P=0.6 Ca=0.84	1.4	8.8	8.8	33
P=0.6 Ca=1.04	1.75	10.0	7.4	40
P=0.6 Ca=1.24	2.07	10.9	6.5	28

\* Tetany

TABLE IV  
EFFECT OF BUTTER FAT 1%, PHOSPHORUS 0.8%, VARIABLE CALCIUM  
OF DIET ON Ca AND P OF SERUM

Diet	Ca/P in diet	Serum Ca	Serum P	Weight gain gm.
P=0.8 Ca=0.24	0.3	6.4	10.2	13
P=0.8 Ca=0.44	0.5	7.2	10.5	6.0
P=0.8 Ca=0.64	0.81	6.8	11.3	29
P=0.8 Ca=0.84	1.05	8.4	8.0	27
P=0.8 Ca=1.04	1.30	8.3	8.7	30
P=0.8 Ca=1.24	1.55	10.5	8.7	23

TABLE V  
EFFECT OF BUTTER FAT 1%, PHOSPHORUS 1.0%, VARIABLE CALCIUM  
OF DIET ON CA AND P OF SERUM

Diet	Ca/P in diet	Serum Ca	Serum P	Weight gain gm.
P=1.0 Ca=0.14	0.14	6.7	11.3	8.0
P=1.0 Ca=0.24	0.24	6.9	11.3	6.0
P=1.0 Ca=0.44	0.44	6.3	10.4	7.0
P=1.0 Ca=0.64	0.64	7.0	10.4	10.0
P=1.0 Ca=0.84	0.84	7.2	9.1	18.0
P=1.0 Ca=1.04	1.04	8.0	8.0	20.0
P=1.0 Ca=1.24	1.24	8.4	7.8	30.0

TABLE VI  
EFFECT OF BUTTER FAT 1%, PHOSPHORUS 1.2%, VARIABLE CALCIUM  
OF DIET ON CA AND P OF SERUM

Diet	Ca/P in diet	Serum Ca	Serum P	Weight gain gm.
P=1.2 Ca=0.14	0.12	5.6	11.5	-5.0
P=1.2 Ca=0.24	0.20	5.9	11.4	+5.0
P=1.2 Ca=0.44	0.37	6.4	9.9	11.0
P=1.2 Ca=0.64	0.54	7.4	9.9	18.0
P=1.2 Ca=0.84	0.7	7.2	9.7	21.0
P=1.2 Ca=1.24	1.04	8.1	—	33.0

an antagonism between calcium and inorganic phosphorus. A high concentration of one element depressed the concentration of the other component in the serum. With the calcium of the diet constant the inorganic phosphorus of the serum fluctuated in a manner parallel with that in the diet. The serum calcium behaved in a similar manner with respect to the

TABLE VII  
EFFECT OF BUTTER FAT 10%, PHOSPHORUS 0.2%, VARIABLE CALCIUM  
OF DIET ON Ca AND P OF SERUM

Diet	Ca/P in diet	Serum Ca	Serum P	Weight gain gm.
P=0.2 Ca=0.145	0.72	7.0	—	31
P=0.2 Ca=0.24	1.22	9.3	8.3	52
P=0.2 Ca=0.44	2.22	10.4	7.0	37
P=0.2 Ca=0.64	3.22	10.9	5.9	41
P=0.2 Ca=0.84	4.22	10.5	3.9	16
P=0.2 Ca=1.04	5.23	10.5	3.5	12
P=0.2 Ca=1.24	6.22	11.0	3.9	12

food calcium. The amount of *available* calcium seems to decrease as the diet phosphorus increases; the same is true of phosphate with increasing amounts of diet calcium. With a diet containing from 0.14 to 0.44 per cent calcium and 0.6 per cent phosphorus a serum calcium of 4.6 to 5.8 mg. was produced with a serum inorganic phosphorus of 10.1 to 12.2 mg. per cent and the animals developed tetany.

#### SERIES 2

A comparison of Tables VII to XII inclusive with the previous tables reveals the effect of the increased content of butter fat in the diet. The effect is presumably due to the increased content of vitamin D in this

diet although the experiments supply no proof for this assumption. With less than optimal amounts of calcium in the diet the calcium level in the serum is higher than in the previous experiments, but still subnormal. Thus, a diet containing 0.24 per cent calcium and 0.2 per cent phosphorus with 10 per cent butter fat gives a serum calcium only slightly less than

TABLE VIII  
EFFECT OF BUTTER FAT 10%, PHOSPHORUS 0.4%, VARIABLE CALCIUM  
OF DIET ON CA AND P OF SERUM

Diet	Ca/P in diet	Serum Ca	Serum P	Weight gain gm.
P=0.4 Ca=0.14	0.36	6.2	11.6	29
P=0.4 Ca=0.24	0.61	7.6	12.0	46
P=0.4 Ca=0.44	1.11	11.0	9.2	42
P=0.4 Ca=0.64	1.61	10.6	11.1	52
P=0.4 Ca=0.84	2.11	10.6	9.5	52
P=0.4 Ca=1.04	2.61	11.0	7.9	46
P=0.4 Ca=1.24	3.11	11.4	9.4	35

normal (9.3 mg. per cent) whereas with 1 per cent butter fat the serum calcium concentration is only 6.2 mg. Similarly the serum inorganic phosphorus level is greater when more butter fat is incorporated in the diet.

### SERIES 3

In this series of experiments cod liver oil was fed with the diet at a 2 per cent level in place of butter fat. With the exception of the last four analyses in Table XIII, where a measurable hypercalcemia seems to have been produced by feeding a diet rich in calcium salts and low in phosphorus, the calcium concentration of the serum maintained a singularly constant level in spite of an approximately nine-fold increase in the

TABLE IX  
EFFECT OF BUTTER FAT 10%, PHOSPHORUS 0.6%, VARIABLE CALCIUM  
OF DIET ON CA AND P OF SERUM

Diet	Ca/P in diet	Serum Ca	Serum P	Weight gain gm.
P=0.6 Ca=0.14	0.24	6.0	9.9	40
P=0.6 Ca=0.24	0.40	8.2	9.3	42
P=0.6 Ca=0.44	0.74	11.1	10.6	54
P=0.6 Ca=0.64	1.07	11.1	9.8	59
P=0.6 Ca=0.84	1.4	10.2	9.2	59
P=0.6 Ca=1.04	1.75	10.5	8.8	60
P=0.6 Ca=1.24	2.07	10.2	8.5	46

TABLE X  
EFFECT OF BUTTER FAT 10%, PHOSPHORUS 0.8%, VARIABLE CALCIUM  
OF DIET ON CA AND P OF SERUM

Diet	Ca/P in diet	Serum Ca	Serum P	Weight gain gm.
P=0.8 Ca=0.14	0.18	5.9	10.4	23
P=0.8 Ca=0.24	0.30	8.5	9.6	30
P=0.8 Ca=0.44	0.55	11.0	9.4	41
P=0.8 Ca=0.64	0.80	10.7	8.8	47
P=0.8 Ca=0.84	1.05	11.4	10.5	48
P=0.8 Ca=1.04	1.30	11.0	9.3	50
P=0.8 Ca=1.24	1.55	10.5	8.8	55

TABLE XI  
EFFECT OF BUTTER FAT 10%, PHOSPHORUS 1.0%. VARIABLE CALCIUM  
OF DIET ON CA AND P OF SERUM

Diet	Ca/P in diet	Serum Ca	Serum P	Weight gain gm.
P=1.0 Ca=0.14	0.14	5.6	13.7	27
P=1.0 Ca=0.24	0.24	8.0	11.0	30
P=1.0 Ca=0.44	0.44	10.7	9.9	43
P=1.0 Ca=0.64	0.64	10.9	9.7	45
P=1.0 Ca=0.84	0.84	11.5	9.0	58
P=1.0 Ca=1.04	1.04	10.7	8.8	55

TABLE XII  
EFFECT OF BUTTER FAT 10%, PHOSPHORUS 1.2%, VARIABLE CALCIUM  
OF DIET ON CA AND P OF SERUM

Diet	Ca/P in diet	Serum Ca	Serum P	Weight gain gm.
P=1.2 Ca=0.14	0.12	5.5	—	8.0
P=1.2 Ca=0.24	0.20	7.0	9.8	28
P=1.2 Ca=0.44	0.37	8.9	9.9	48
P=1.2 Ca=0.64	0.54	9.9	9.9	49
P=1.2 Ca=0.84	0.70	10.7	9.9	54
P=1.2 Ca=1.04	0.87	10.9	10.0	55
P=1.2 Ca=1.24	1.04	11.8	10.6	53



TABLE XIII  
EFFECT OF COD LIVER OIL 2%, PHOSPHORUS 0.2%, VARIABLE CALCIUM  
OF DIET ON Ca AND P OF SERUM

Diet	Ca/P in diet	Serum Ca	Serum P	Weight gain gm.
P=0.2 Ca=0.14	.72	10.0	11.8	63
P=0.2 Ca=0.24	1.20	10.6	13.1	53
P=0.2 Ca=0.44	2.22	10.9	9.0	52
P=0.2 Ca=0.64	3.22	12.6	6.8	42
P=0.2 Ca=0.84	4.22	13.2	7.5	23
P=0.2 Ca=1.04	5.22	13.3	8.1	13
P=0.2 Ca=1.24	6.22	13.6	7.5	10

TABLE XIV  
EFFECT OF COD LIVER OIL 2%, PHOSPHORUS 0.4%, VARIABLE CALCIUM  
OF DIET ON Ca AND P OF SERUM

Diet	Ca/P in diet	Serum Ca	Serum P	Weight gain gm.
P=0.4 Ca=0.14	0.36	10.7	9.3	72
P=0.4 Ca=0.24	.61	10.6	9.0	75
P=0.4 Ca=0.44	1.11	10.6	8.6	81
P=0.4 Ca=0.64	1.61	10.6	9.3	62
P=0.4 Ca=1.24	3.11	12.0	9.1	77

calcium of the diet and a six-fold variation of the phosphorus in the diet. Similarly disregarding the hyperphosphataemia in the first three analyses in Table XV and four in Table XVI we see here also a remarkable constancy of the inorganic phosphorus in the serum.

#### GROWTH

The rats were weighed at the beginning and end of the experimental period. The recorded gain in weight represents an average of the weight gain of five to eight animals. In most of the experiments eight animals were weighed and the increase averaged. Marked deviations from the average were observed in single litters.

With a diet containing only 1 per cent butter fat and 0.14 per cent calcium the gain in weight decreased as the phosphorus in the diet was increased. With moderate amounts of calcium in the diet, *i.e.*, 0.64 per cent, the weight gain again dropped as the phosphorus of the diet increased, the increase of food phosphorus being equal to a drop in diet calcium. With the addition of 10 per cent butter fat to the diet there was a distinct improvement in the weight gain for each group. The substitution of cod liver oil still further improved the weight gain of all the animals.

In the experiment summarized in Table XIII we find a progressive decline in weight gain as the calcium increased in the diet. A similar phenomenon though less striking is seen in Table VII in the corresponding experiment with butter fat 10 per cent. In these experiments the low food phosphorus in the presence of a high calcium intake acts as the limiting factor for growth and the higher the calcium intake with a fixed concentration of phosphorus in the diet the poorer the growth.

Failure to gain weight due to low calcium intake is more readily overcome by addition of cod liver oil than is that due to inadequate phosphorus intake. Thus, with a diet calcium of 0.14 per cent and a diet phosphorus of 0.2 the weight gain with 1 per cent butter fat is 21 gm., that with 10 per cent butter fat is 31 gm. while that with 2 per cent cod liver oil is 63 gm. Whereas with a calcium intake of 1.24 per cent and a phosphorus in the diet 0.2 per cent there is very little difference in weight gain whether 1 per cent butter fat, 10 per cent butter fat or 2 per cent cod liver oil is used, namely, 20, 12, 10 gms. respectively. That lack of *available* phosphorus in the diet is the limiting factor in growth even in the presence of 10 per cent butter fat or 2 per cent cod liver oil is indicated by the fact that a marked improvement in weight follows the addition of phosphorus to the diet, other factors remaining constant. An increase of but 0.2 per cent phosphorus in the diet has a striking effect.

TABLE XV  
EFFECT OF COD LIVER OIL 2%, PHOSPHORUS 0.6%, VARIABLE CALCIUM  
OF DIET ON CA AND P OF SERUM

Diet	Ca/P in diet	Serum Ca	Serum P	Weight gain gm.
P=0.6 Ca=0.14	.24	11.2	12.2	54
P=0.6 Ca=0.24	.40	11.2	11.1	73
P=0.6 Ca=0.44	.74	10.3	10.8	55
P=0.6 Ca=0.64	1.07	10.2	9.0	87
P=0.6 Ca=0.84	1.40	10.5	8.5	55
P=0.6 Ca=1.04	1.74	10.8	9.1	73
P=0.6 Ca=1.24	2.07	10.6	8.3	65

TABLE XVI  
EFFECT OF COD LIVER OIL 2%, PHOSPHORUS 0.8%, VARIABLE CALCIUM  
OF DIET ON CA AND P OF SERUM

Diet	Ca/P in diet	Serum Ca	Serum P	Weight gain gm.
P=0.8 Ca=0.14	.18	9.6	9.5	60
P=0.8 Ca=0.24	.30	10.2	10.	65
P=0.8 Ca=0.44	.55	10.3	11.3	55
P=0.8 Ca=0.64	.80	10.2	10.7	52
P=0.8 Ca=0.84	1.05	10.2	11.4	56
P=0.8 Ca=1.04	1.30	10.9	8.4	70
P=0.8 Ca=1.24	1.55	10.2	8.5	83

TABLE XVII  
EFFECT OF COD LIVER OIL 2%, PHOSPHORUS 1.0%, VARIABLE CALCIUM  
OF DIET ON CA AND P OF SERUM

Diet	Ca/P in diet	Serum Ca	Serum P	Weight gain gm.
P=1.0 Ca=0.14	0.14	10.2	7.5	71
P=1.0 Ca=0.24	0.24	10.0	8.0	65
P=1.0 Ca=0.44	0.44	9.8	7.6	70
P=1.0 Ca=0.64	0.64	10.0	7.5	73
P=1.0 Ca=0.84	0.84	10.2	8.5	83
P=1.0 Ca=1.04	1.04	10.3	8.3	68

TABLE XVIII  
EFFECT OF COD LIVER OIL 2%, PHOSPHORUS 1.2%, VARIABLE CALCIUM  
OF DIET ON CA AND P OF SERUM

Diet	Ca/P in diet	Serum Ca	Serum P	Weight gain gm.
P=1.2 Ca=0.14	.12	10.7	9.5	53
P=1.2 Ca=0.24	.20	10.3	8.8	53
P=1.2 Ca=0.44	.37	10.9	9.3	69
P=1.2 Ca=0.64	.54	10.7	8.4	61
P=1.2 Ca=0.84	.70	10.5	8.2	71
P=1.2 Ca=1.04	.87	10.2	8.5	67
P=1.2 Ca=1.24	1.04	10.8	8.9	58

TABLE XIX  
EFFECT OF VARIABLE AMOUNTS OF CALCIFYING FACTOR WITH CALCIUM  
AND PHOSPHORUS IN THE DIET AT CONSTANT AND MINIMAL LEVELS

Diet	Vitamin	Ca	P
P=0.181 Ca=0.045	C. S. oil 10	6.2	9.3
P=0.181 Ca=0.045	B. Fat 1.0	5.8	10.0
P=0.181 Ca=0.045	No Fat	6.0	9.1
P=0.181 Ca=0.045	B. Fat 10.0	6.0	12.0
P=0.181 Ca=0.045	C. L. O. 2%	7.2	5.6

C. S. = Cotton Seed Oil

B. F. = Butter Fat

C. L. O. = Cod Liver Oil

#### Series 4

In this series of experiments diets containing minimal amounts of calcium and very small amounts of phosphorus were fed. These diets were fed either alone or with a supplement containing a variable amount of vitamin D. In this experiment neither cotton seed oil nor butter fat fed at 1 or 10 per cent level had any influence upon the serum calcium. It is significant that in the presence of minimal amounts of calcium and phosphorus in the diet enough phosphorus was absorbed to produce hyperphosphataemia in the absence of calcifying factor but the serum calcium remained low. Two per cent cod liver oil, however, not only raised the serum calcium but in doing so depressed the inorganic serum phosphorus.

#### Series 5

In this series of experiments, the calcium and phosphorus concentrations in the diet were maintained at a constant level as was the calcium/phosphorus ratio but the fat soluble factor was varied. When this Ca/P ratio in the diet is 1.5, the same calcium and inorganic phosphorus prevails in the serum irrespective of the amount of organic factor in the diet. Although the calcium/phosphorus ratios in the diets in this series of experiments varied from Ca/P=0.4 to Ca/P=6.0, 2 per cent cod liver oil added to the diet served to maintain a normal concentration of calcium and inorganic phosphorus in the blood serum.

TABLE XX  
EFFECT OF VARIABLE VITAMIN D IN DIET

Diet Ca/P=0.4		P=1.0	Ca=0.44
Vitamin D	Serum Ca	Serum P	Weight gain gm.
B.F. 1	6.3	10.4	7.0
B.F. 10	10.7	9.9	43
C.L.O. 2	9.8	7.6	70
Diet Ca/P=1.0		P=1.0	Ca=1.04
B.F. 1	8.0	8.0	20
B.F. 10	10.7	8.8	55
C.L.O. 2	10.3	8.3	68
Diet Ca/P=1.5		P=0.8	Ca=1.24
B.F. 1	10.5	8.7	23
B.F. 10	10.5	8.8	55
C.L.O. 2	10.2	8.5	82
Diet Ca/P=6.0		P=0.2	Ca=1.24
B.F. 1	10.2	3.0	20
B.F. 10	11.2	3.9	12
C.L.O. 2	13.6	7.5	10

### DISCUSSION

The maintenance of a normal concentration of calcium and of inorganic phosphorus in the serum is an example of biological regulation. The maintenance of a fairly constant body temperature, in the warm blooded animal, of a fixed hydrogen ion concentration in the blood, and of a constant osmolar concentration in the serum are other examples of such regulation. These phenomena involve the integration of biological and purely physico-chemical factors for the preservation of certain physiological equilibria. Various factors play a part in this regulatory mechanism. The concentration of calcium and phosphate in the diet, its content of vitamin D, as well as the concentration of other salts, the amounts of organic factors such as fat, carbohydrate and protein, and the reaction which the diet yields ultimately in the body or in the gastro-intestinal tract, all play a part in determining the absorption of bone forming salts. Endocrinological factors may determine the ebb and flow of calcium salts from and into the tissues or their elimination by the excretory organs.

Physico-chemical factors undoubtedly control in part the level at which calcium and inorganic phosphorus are maintained in the blood.

#### ABSORPTION AND EXCRETION OF CALCIUM SALTS

The literature dealing with the absorption and excretion of calcium salts has been critically reviewed by Stewart and Percival (19) and hence the interested reader will be referred to this article for details. The form in which the calcium is present in the diet in all probability affects its availability. Other facts may play a part. Only three variables have been studied. In these experiments the basal diet was kept constant and only the calcium, the total phosphorus and the fat soluble factor were varied.

In the absence of all but minimal amounts of vitamin D in the diet, physico-chemical factors seem to have full sway in the gastro-intestinal tract. Under these conditions insoluble or slightly soluble salts of calcium are less readily absorbed than soluble ones (20). At the reaction which prevails normally in the different parts of the small intestine of the rat; pH 5.2 to 6.5 (21), the less soluble phosphates of calcium,  $\text{CaHPO}_4$  and  $\text{Ca}_3(\text{PO}_4)_2$  as well as insoluble calcium soaps may be found in different proportions depending upon the pH of the intestinal contents, their content of phosphates, carbonates and fatty acids, the concentration of other salts, and possibly on other as yet undetermined factors.

With large amounts of Ca in the diet and minimal amounts of phosphorus, *i.e.*, high Ca/P ratio, much of the phosphate is precipitated as insoluble phosphate and is either very gradually absorbed or re-excreted in the feces without ever having actually entered the blood.<sup>3</sup> The excess of calcium is absorbed and produces a normal or even an excessive concentration of serum calcium. The failure of absorption of phosphate aided by the actual abstraction of phosphorus from the blood by the excess of Ca in the bowel, produces a lowering of the inorganic serum phosphorus. Although calcium was fed for the most part as  $\text{CaCO}_3$ , much of this was undoubtedly converted into chloride in the stomach while at the pH prevailing in the intestine it must have remained at least in part as  $\text{Ca}(\text{HCO}_3)_2$ ,  $\text{CaHPO}_4$ ,  $\text{Ca}[\text{H}_2\text{PO}_4]_2$  and  $\text{Ca}_3(\text{PO}_4)_2$ , a small fraction remaining as  $\text{CaCO}_3$  and calcium soaps. A similar mechanism may be responsible for the high inorganic serum phosphorus with low calcium and high

<sup>3</sup> One cannot conclude from the presence of calcium in the stools that ingested calcium has not been absorbed. Calcium may be absorbed and re-excreted into the intestine, when physical and chemical factors in the urine prevent its excretion by the kidney. That calcium derived from bone may find its way into the intestine is indicated by the excretion of amounts of calcium in the stools in excess of the amount ingested when animals and humans are fed a calcium poor diet.

phosphorus in the diet. After combining with Ca to form insoluble calcium phosphate the excess of  $\text{HPO}_4$  and  $\text{H}_2\text{PO}_4$  and their salts may remain. These are in turn absorbed and raise the inorganic serum phosphorus concentration above the normal level.

The work of Schabad (22) and of E. Schloss (23) has shown that the absorption of calcium phosphate is favored by the addition of cod liver oil. Even very small amounts of calcium in the diet suffice to maintain a normal concentration of this element in the serum. This holds true also for phosphate. The added fat, as such, does not account for this phenomenon. Vitamin D administered as ultra violet light (24) or irradiated ergosterol or as irradiated milk (25) acts in a similar manner. Not only may less calcium and phosphorus appear in the stools and more in the urine, indicating improved absorption of these elements, but more is retained. In fact the amounts retained are so large as to justify the conclusion that the excess is deposited in the bones, a conclusion which in the case of rachitic children can be verified by x-ray, or in experimental animals by analysis of the bones.

Cod liver oil, therefore, facilitates both the absorption and deposition of bone salts and in this manner regulates calcium and inorganic phosphorus metabolism. Vitamin D, the active principle concerned in this function, is more than an anti-rachitic factor since it exerts a controlling influence on calcium and phosphorus metabolism irrespective of whether rickets is present or not. Where the relative concentration of these elements in the diet is optimal for the animal so that normal concentrations of serum calcium and inorganic phosphorus prevail even with minimal amounts of vitamin D in the diet, the value of the cod liver oil addition is shown in the greater weight gain of the animals receiving the oil.

It is worthy of note that the minimal amounts of phosphorus absorbed under the influence of cod liver oil from low phosphorus, high calcium diets suffice to maintain a normal serum phosphorus level but are insufficient to allow normal gain in weight. There is not enough extra phosphorus for skeletal growth or the formation of protoplasm. Cod liver oil brings about the economical utilization of calcium and of phosphorus but it cannot wholly replace these substances; a certain minimum is essential.

At least two factors prevent the accumulation of calcium and phosphorus in the blood thus avoiding hypercalcaemia, hyperphosphataemia or both, namely the excretion of the excess by the kidneys or by the bowel or the deposition of these materials as insoluble calcium phosphate in the bones. The formation of insoluble calcium phosphate serves as a buffer preventing the excessive concentration of these elements in serum just as



the formation of weakly dissociated acids and their alkaline salts prevents the increase in the hydrogen ion concentration when strong acids are added to serum. Cod liver oil enhances the efficiency of the mechanism by facilitating absorption of calcium phosphate on the one hand and the deposition of the insoluble phosphate and carbonate of calcium on the other.

Cod liver oil increases the availability of both calcium and phosphorus and enables the body to function more economically with respect to these salts. The serum calcium concentration is restored to normal and excellent gain in weight is obtained even with minimal amounts of diet calcium. However, when diets containing minimal amounts of phosphorus are fed, the same amount of cod liver oil restores the serum inorganic phosphorus to the usual value but there is little growth. An increase of 0.2 per cent in food phosphorus increases the weight gain for the experimental period from 10 gm. to 77 gm. Further increase of diet phosphorus does not materially influence the concentration of inorganic phosphorus in the serum. A deleterious effect upon the weight is discernable from a large excess. Both serum calcium and inorganic phosphorus *values remain unaltered.*

But why should the normal concentration of calcium and of phosphate ion remain at a constant level in the serum, under normal conditions, and why must the level for the normal rat be approximately 10 mg. per cent of Ca = 2.5 mMol. of calcium and 8 mg. per cent of inorganic P or 2.6 mMol. of phosphorus? Undoubtedly physico-chemical factors determine this level. Calcium is present in serum as ionic calcium  $\text{Ca}^{++}$ , as Ca bound to non-diffusible ions, probably protein acids and as slightly dissociated but diffusible calcium salts. The relative proportion of these forms has only been determined approximately in the normal animal and varies in disease. Inorganic phosphorus at the pH of blood serum exists as  $\text{PO}_4^{=}$ ,  $\text{HPO}_4^{=}$  and  $\text{H}_2\text{PO}_4^-$ . The equilibrium between these ions is dependent upon the pH of the serum, its total ionic strength, its temperature and probably other factors. It is possible that a dynamic equilibrium exists between the ions, calcium and phosphate, and the same ions in the tissue fluid bathing the bony trabeculae. There is some evidence that  $\text{CaHPO}_4$  (25) plays an intermediary role in the deposition of calcium phosphate and recent, analytical and spectroscopic studies indicate that the inorganic matter of bone contains both  $\text{Ca}_3(\text{PO}_4)_2$  and another calcium salt [Ca X]. Normal sera which have a calcium concentration of Ca = 2.5 mM and P = 5.0 mg. per cent or about 1.6 mM are in equilibrium with  $\text{CaHPO}_4$ . Higher concentrations are possible through the formation of diffusible or non-diffusi-

ble unionized but soluble compounds of calcium or phosphate. This explanation has been advanced by Greenwald (27) to explain the hypercalcaemia after overdosage with parathyroid extract and direct proof of the existence of soluble, non-diffusible weakly ionized, soluble compounds of calcium and citrate has been supplied by Shear and Kramer (28). Evidence for the clinical importance of such compounds has been supplied by Shelling and Maslow (29). The maintenance of a normal concentration of serum calcium and inorganic phosphorus is therefore the resultant of the rate of absorption of these elements from the gastro-intestinal tract, their rate of deposition in the tissues, particularly the bones, and the rate of excretion by the bowel and the kidneys and of certain physico-chemical conditions in the blood.

#### CONCLUSIONS

In these experiments the concentration of calcium and inorganic phosphorus in serum varied with the concentration of these components in the diet and with the amount of vitamin D. With minimal amounts of vitamin D the calcium of the serum varied directly as the calcium concentration in the diet. The same was true as regards the phosphorus of the serum. Increasing the calcium in the diet increased the calcium in the serum and depressed the phosphorus. The opposite effect was obtained when the phosphorus of the diet was increased. In the latter case, the calcium concentration was reduced to a minimal value unless an adequate amount of calcium was given to counteract this effect.

Deviations of the calcium and inorganic phosphorus of the serum from the normal, produced by marked disproportion in the concentration of these elements in the diet were less marked as the concentration of vitamin D in the diet increased. Vitamin D stabilizes the calcium and inorganic phosphorus concentrations in the serum. Both hypercalcaemia and hyperphosphataemia may be produced by appropriate dietary measures.

A Ca/P ratio in the diet of 1.5 (1.2 gm. of calcium and 0.8 gm. of phosphorus) gave the same value for the concentrations of these components in the serum, *i.e.*, normal values, irrespective of the amount of vitamin D. The effect of the vitamin D was evident in the superior gain in weight.

The calcium or phosphorus concentration of the diet, the Ca/P ratio, or the fat soluble calcifying factor may be the limiting factor in growth.

The regulatory influence of cod liver oil upon calcium and phosphorus metabolism has been confirmed.

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# A TECHNIC FOR STUDYING LACTATION IN SMALL ANIMALS AND ITS USE IN EVALUATING PROTEIN LEVELS IN THE DIET

By

MARJA KOZLOWSKA, C. M. McCAY, AND L. A. MAYNARD

*(From the Laboratory of Animal Nutrition, Cornell University, Ithaca)*

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IN ATTEMPTING to improve the current technic for studying the nutritional requirements of lactating rats, we have tested the three following possibilities: 1.—The prolongation of the lactation period of the mother by forcing her to adopt a series of new litters of animals, each younger than the preceding. This method was used previously in a few experiments by Maeder (1). This technic was abandoned after some preliminary trials due to the failure to get uniform results, the difficulty of getting all mother rats to adopt strange young litters and the complication of providing a regular supply of younger animals of the correct age. 2.—The feeding of the mother by mechanical devices that permit her to eat, leaving the young dependent solely upon the mother's milk before and after the weaning period. We have failed in this method thus far because the young rats have outwitted us in every attempt to develop a device that provides the mother with feed and excludes the young. 3.—The removal of the mother from the cage for separate feeding. This allows regular consumption of feed and regular periods for nursing the young. All the studies of this report were made with rats and employed this technic. Various feeding schedules were tried. The following proved satisfactory: 6 to 7 a.m., 9 to 10 a.m., 12 m. to 1 p.m., 3 to 4 p.m., 6 to 7 p.m., 9 to 10 p.m. Occasionally the last feeding at night was prolonged since the animal was more inclined to eat at this time. Among other schedules tested was one with an additional period from 3 to 5 a.m. This gave slightly better results but hardly justified the additional labor.

This technic which we have adopted rests upon the assumption that a relation exists between the food consumed by the animal and the milk secreted during a period of prolonged lactation. The advantage of this method is that it is possible to employ rats for lactation studies, to feed them synthetic diets and to put pressure upon the lactating mother for an optimum secretion of milk during a prolonged period. Thus we tend to exhaust effective stores of nutrients that may function for limited periods and give false results in short period studies. The chief disadvantages of

the method are the labor involved, and the large number of breeding females that must be withdrawn from the production of young in the stock colony. A third disadvantage that we have been unable to eliminate is the consumption of the mother's feces by the young. We have kept the litters upon false bottom cages with meshes sufficiently large to drop the feces, but some eating of them has been observed.

In order to get uniform results, we have used five mother rats with litters of the same age upon each experimental diet. To secure further uniformity we have provided litters of six rats for each mother by a suitable exchange of animals while quite young. In one series of experiments, Chart 2, the young of every mother were reduced to five per litter in order to determine if six animals consumed all the milk possible.

To test the suitability of the technic we have considered only one variable, namely the protein. The following are two typical diets we used:

	High protein	Low protein
Casein (commercial American) . . . . .	40	10
Calcium carbonate . . . . .	1	1
Bone meal . . . . .	1	1
Sodium chloride . . . . .	1	1
Lard . . . . .	15	15
Yeast . . . . .	5	5
Cod liver oil . . . . .	5	5
Cooked starch . . . . .	32	62

In order to insure an adequate supply of supplements, each mother was fed an additional half gram of dried yeast daily. Since we were primarily concerned in developing a technic, we have not worked with purified constituents. On the other hand we have worked with a diet composed of materials that lend themselves to limited purification in preference to such unknowns as dried meats or cereal grains. We have probably provided more yeast than necessary but wished to supply an adequate amount to prevent the B factors from introducing uncontrolled variables. In one series of experiments the young were fed yeast and cod liver oil separately with a medicine dropper, but since a limited trial did not alter the growth curves, this technic was not adopted.

In the preliminary trials it was found that the young maintained upon the milk of the mother grew very anemic. In the experiments reported this was prevented by administering small amounts of iron citrate, copper sulfate and potassium iodide to the young before they started to consume water. To each ten liters of water provided in the cages were added 3.5 gm. iron citrate, 0.1 gm. copper sulfate, and 0.008 gm. potassium iodide. This

afforded ample protection after the young started drinking. The young rats under these conditions did not become anemic.

In Chart 1 we have shown the mean food consumption of five mother rats upon the high protein diet and five upon the low level. We have also plotted the mean growth curves for the thirty young rats in each group. The curves are plotted upon semi-log paper and the probable error curves are shown on each side of the mean weight curve. The broken portion of the curves represents the periods when the young were dying. Photograph 1

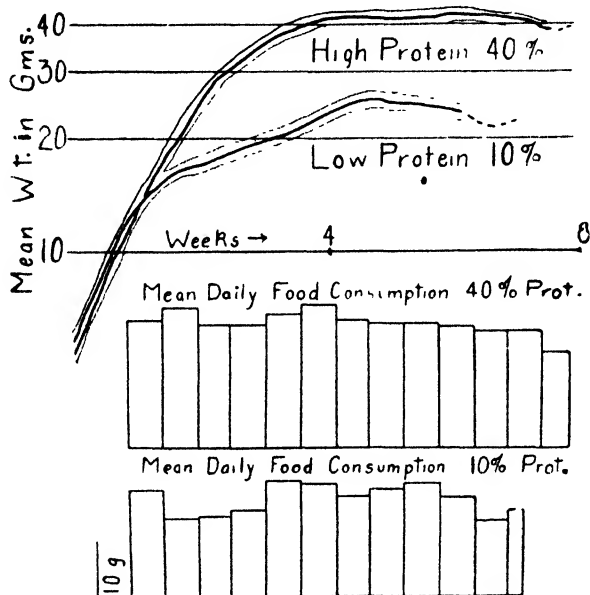


CHART 1. —Mean growth curves for thirty young rats nursed by mothers fed a high protein diet and for a similar number nursed by mothers upon a low protein intake. The food intake data represent the mean consumption per day per mother considered in time units of four days each.

shows the relative size of the rats just before the termination of the experiment. One typical young rat from each litter was selected for the picture.

In Chart 2 we have shown a similar experiment in which the protein levels were brought nearer together by using the same diets and exchanging casein for starch.

These data indicate that the technic is reliable in detecting the effect of different protein levels upon the secretion of milk. But the mothers upon the higher protein levels consumed more food. This contrasts with the observations of Adair (2) who found that nursing women upon high protein diets consumed less food. Since we felt the increased secretion of milk upon

the high protein diet might be merely the result of consuming more calories, we carried out a new series in which the mothers upon the high protein levels were limited in food intake to that consumed by those upon the lower levels. The result of this series is shown in Chart 3 and Photograph 2. From this last experiment one must conclude that the protein was responsible for the differences observed.



PHOTOGRAPH 1.—The relative size of rats nursed by mothers upon high and low protein diets

The graphs show that lactating mother rats are unable to rear litters of six young much if any above the weight attained at the time of normal weaning. After attaining such a weight, say, about forty grams, they can be maintained upon the mother's milk until they are fifty or sixty days of age. At this time the milk supply seems to fail gradually. There is a considerable variation in mothers, however, and some litters were killed at the end of two months merely because other litters in the comparison had failed. Some mothers could have maintained their young for a much longer period.

The weight attained by the rats of Graph 2 is greater than that found in 1. This is due to the fact that only five animals were used in each litter

for the second experiment. We feel that this shows that six normal young rats take all the milk the mother secretes. When there are only five in a

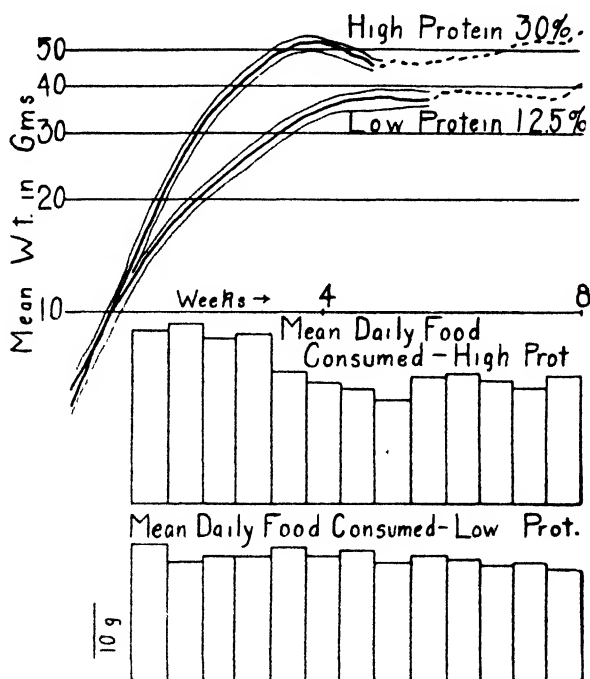


CHART 2. Mean growth curves for nurslings when the number per litter is five instead of six and when the protein levels are close together.

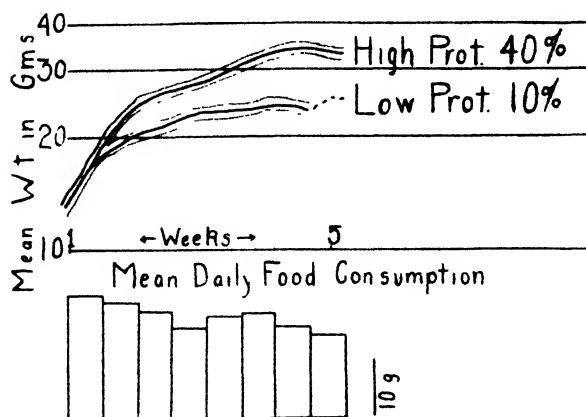
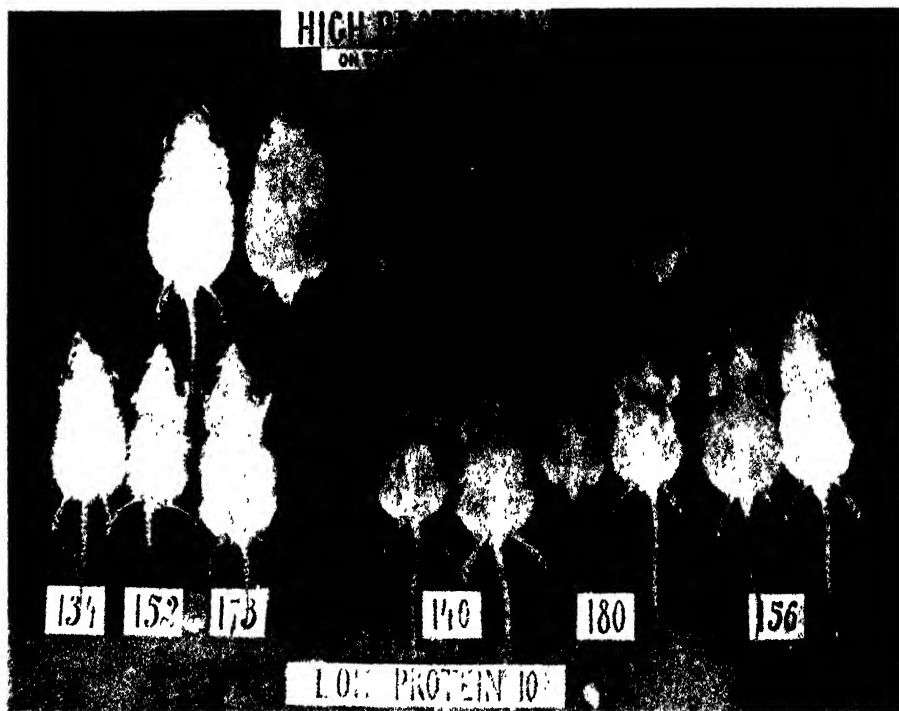


CHART 3.—Mean growth curve for thirty nurslings reared by five mothers upon high protein diets, contrasted with that for a similar group upon a low protein diet. The mean food intake per mother was the same in each group and was determined by the consumption of those upon the lower protein diet.



litter they attain a greater weight because there is more milk available per nursing.

Upon the high protein diets the mothers either gained weight or maintained their initial body weight, while at the lower levels there was a sacrifice of body tissue. These results do not conflict with those of Maynard and Bender (3) since casein alone cannot be considered a source of protein equal in value to several mixed proteins.



PHOTOGRAPH 2.—The relative size of rats nursed by mothers allowed equal intakes of high and low protein diets.

#### SUMMARY

A technic for determining the nutritional factors that control the secretion of milk in small animals has been devised. It is based upon the assumption that the prolongation of the lactation period may serve as a measure of nutritional factors. In this method litters are reduced to six in number. The nursing mother is allowed alternate periods for feeding and suckling her young. Anemia is prevented from developing in the young by feeding small amounts of iron, copper, and iodine before the animals begin to drink and by placing these elements in the drinking water when the young are

older. Under such conditions mothers can rear the young to about the weight of normal weaning and can maintain them until they are about two months old. With litters of five the weight attained is somewhat greater, indicating that six young consume all the milk secreted. There is a considerable individual variation among mothers. Some can extend their lactation period beyond the two months studied.

Casein fed at ten and forty per cent levels showed the lower one to be quite inferior. The nursing mothers consumed more food upon the higher protein level when they were allowed an unrestricted intake. In one series the intakes of the higher protein group were made the same as those upon the lower. The higher protein diet still produced superior results. Since much more work must be done before the answer to this protein problem can be given, we are presenting these experiments chiefly as an illustration of the technic.

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## PROTEIN INTAKE AND BASAL METABOLISM OF COLLEGE WOMEN\*

BY ROSSLEENE ARNOLD HETLER

With the assistance of Marie Killinger and Margaret Plant

*(From the Nutrition Laboratory of the Home Economics Department,  
University of Illinois, Urbana)*

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**A**LTHOUGH a number of experiments have been carried on in an attempt to find out the protein intake and protein requirement of men, very little effort has been made to investigate the actual protein consumption and protein requirement of women. The present investigation was conducted on a group of healthy college women for the purpose of determining their habits of protein intake. Basal metabolism tests were also carried on and a study was made of a possible relationship between the quantity of protein ingested and the basal metabolic rate.

Sherman (1) in 1920 reported studies on the protein of the diets of adults who showed normal health and digestion. Using the total output of nitrogen as an indication of protein requirement, data were summarized from 109 experiments. Although 67 experiments were performed on men and 42 on women, only eight of the 37 subjects considered were women. The calculations showed that the average protein requirement for men or for women was practically identical when averaged to the same basis of body weight. The protein requirement was found to range from 21 to 65 grams with an average of 44.4 grams per 70 kilogram man per twenty-four hours. From these findings Sherman made the following statement concerning the protein needs in adult human nutrition, "A standard allowance of one gram per kilogram or one-half gram per pound per day appears to provide a margin of safety of 50 to 100 per cent as far as adult human nutrition is concerned." This standard allowance of protein supports the earlier view of Chittenden (2), who decided from his experiments on men and on dogs that the usual protein intake of men was far too high and that 60 grams or less of protein per day in the diet of a 70 kilogram man was ample for the nitrogenous needs of the body. In recent years reports have been made showing the nitrogen elimination of medical students. Brooks (3) in 1929 reported a study of the nitrogen excretion of 192 male medical students. Beard (4) in 1927 presented data for the nitrogen excretion of 400 male medical students. Denis and Borgstrom (5) in 1924 reported a study of a group of 242 medical students, including 9 women and 233 men. The nitrogen excretion per kilogram body weight was about the same for the women as for the men. The protein intake of individuals in these groups of medical students was shown to average slightly more than one gram of protein per kilogram body weight and thus satisfies the Sherman standard of protein consumption. The nitrogenous intake of these individuals is, however, far below that suggested by Pearl (6) in his report of 1920 and less than that recommended by Atwater (7) in 1895 and Benedict (8) in 1906. In a recent review of the question of protein intake, Mitchell (9) makes the following statement, "There are communities and races of men that have subsisted for generations on small amounts of protein

\* Data for some of the experiments here reported were taken from the thesis submitted by Marie Killinger, in partial fulfillment of the requirements for the Degree of Master of Science in Home Economics in the Graduate School of the University of Illinois, 1929.

comparable to those advocated by Chittenden and Hindhede, but their physical, intellectual, and industrial characteristics are not such as to inspire confidence in the wisdom of their habits or any desire to follow in their footsteps." Mitchell concludes that with protein, as with other nutrients of the diet, it is possible to maintain nutritional health and physical efficiency over a wide range of intake.

In a series of observations made by the writer in relation to the protein intake of some of the Home Economics students at Iowa State College in 1927, it was found that the daily ingestion of protein was sometimes as high as 70 grams but more often it was as low as 25 to 40 grams and the average protein consumption of the group studied was somewhat less than the standard set by Sherman. Data for some of these observations were obtained from determinations of the excretion of nitrogen but more often the protein intake for this group of individuals was calculated from dietary records of food intake. The degree of accuracy of the data was thus uncertain but it appeared that the dietary habits of these college women were such that the protein intake was probably less than one gram per kilogram body weight. Further study of the amount of protein ingested by college women was carried on at the University of Illinois during the next three years. Determinations were made of the urinary nitrogen, basal metabolism, and protein and caloric intake of 85 women students. The majority of the subjects were juniors, seniors, or graduate students in the Home Economics department and most of them were interested in nutrition. These students lived the active routine of campus life and were healthy individuals whose weight approached normal and remained for the most part constant throughout the year. The group of individuals studied ranged in age from 19 to 37 years but 85 per cent of them were between 19 and 24 years of age. They weighed from 42.7 to 82.8 kilograms and the average weight for the group was 56.8 kilograms. The subjects offered their coöperation in this study and were extremely careful to record their food intake and to eat according to their usual habits during the days of the observations. The group studied included women who had their meals at sorority houses, at dormitories, at restaurants, and at home. The subjects recorded the kind and approximate amount of food eaten daily for at least one period of three days to a week. Very often the individuals served for two and sometimes three periods at different times of the year. An approximate estimate of the calories and protein of the diet was made and from the latter the nitrogen was calculated and compared with the total nitrogen excretion. At least two and usually three or more 24-hour urine specimens from each subject were analyzed for total nitrogen, creatinine, and acidity. A total of 320 twenty-four-hour urines from 85 women students

were analyzed. In some cases the analysis was made by the senior students but many of the determinations were carried on by Killinger.

Basal metabolism determinations were made on each of the 85 students of the group. These tests were made by Killinger or by Plant or by students whose work was under the direction of Plant. The Benedict-Roth metabolism apparatus was used and the per cent deviation of the actual basal metabolism from the normal prediction was calculated according to the Harris-Benedict standard. Three six-minute test periods were taken for each determination and at least two and sometimes three or more determina-

TABLE I

TOTAL NITROGEN EXCRETION IN THE URINE AND BASAL METABOLISM AVERAGED ACCORDING TO YEARS IN WHICH THE OBSERVATIONS WERE MADE

Year	No. of subjects	Average weights	Average urinary nitrogen per 24 hours	Average deviation of actual basal metabolism from Harris-Benedict prediction
		kg.	gm.	per cent
1928	21	59.5	7.81	-4.5
1929	27	55.1	8.66	-8.9
1930	37	56.6	6.84	-7.3
Average	85	56.8	7.69	-7.1
Average per 70 kg. man equivalent			9.48	

tions were made usually on successive days and always in the inter-menstrual period. The basal metabolism determinations were, as a rule, made on the same days upon which the 24-hour urines were collected. The tests were made before breakfast and in each case a one-half hour rest period preceded the determination. The oral temperature and the pulse rate were always taken.

In Table I are shown the averages of the results of the analyses of the 320 urine specimens. The averages of the results of the basal metabolism tests are also given. The urine analyses showed that the daily excretions ranged from 3.3 to 11.8 grams of nitrogen although the majority of the eliminations were between 6.4 and 8.9 grams. The average excretion for the group, as the table shows, was 7.69 grams of nitrogen. Since the average weight of the group was 56.8 kilograms, the equivalent 24-hour excretion for a 70 kilogram man would be 9.48 grams of nitrogen. Denis and Borg-

strom (5) at Tulane University found the average excretion of the group calculated for a 70 kilogram individual to be 11.07 grams nitrogen. Beard (4) at Western Reserve University found 11.16 grams nitrogen as the equivalent for a 70 kilogram man. And Brooks (3) at the University of North Carolina obtained 10.43 grams nitrogen as the equivalent 24-hour excretion for a 70 kilogram individual. These values are all higher than the 9.48 grams, the equivalent nitrogen excretion for a 70-kilogram person, calculated from our data on women students. When 10 per cent is added for protein loss through fecal nitrogen, the protein equivalent for a 70 kilo-

TABLE II

COMPARISON OF THE AVERAGE PROTEIN EQUIVALENT OF THE NITROGEN EXCRETION, AND OF THE BASAL HEAT PRODUCTION WITH THE ESTIMATED PROTEIN AND CALORIC INTAKE OF COLLEGE WOMEN

No. of subjects	Average weight	Average urinary nitrogen	Average protein equivalent of urinary nitrogen (adding 10 per cent for loss in feces)			Average total heat production per 24 hours (Benedict)	Food intake (estimated from diet records)		
							Average protein intake		Average caloric intake
			per kg. body wt.	per 56.8 kg. body wt.	per 70 kg. man equivalent		per kg. body wt.	per 56.8 kg. body wt.	
85	kg. 56.8	gm. 7.69	gm. 0.94	gm. 53.4	gm. 65.8	cal. 1260	gm. 0.97	gm. 55	cal. 1700

gram individual, as shown in Table II, is 56.8 grams or 0.94 grams per kilogram body weight. The average protein intake for the women students is thus shown to be slightly less than one gram per kilogram body weight while the average protein intake of male medical students was shown by Denis and Borgstrom, by Beard, and by Brooks to be slightly more than one gram per kilogram body weight. It thus appears that the protein intake of the average college woman is probably somewhat lower per kilogram body weight than that of male medical students, and slightly below the Sherman standard.

The average basal metabolism of the 85 women in the group studied, as Table II shows, was 1260 calories per 24 hours. When this is calculated in terms of deviation from the normal, the average deviation of the actual basal metabolism from the Harris-Benedict prediction is, as Table I shows, -7.1 per cent. The deviations from standard for the various

individuals studied ranged from  $-20.0$  to  $+11.0$  per cent. Only 12 of the subjects showed a basal metabolism which was above the predicted value. All others showed metabolism values below the normal prediction. But of those whose metabolism was below the normal prediction only 17 showed an average deviation between the standard and  $-5.0$  per cent. Thus even if, as Benedict (10) suggests, it is accepted that the standards are five per cent too high for women, over half of this group shows a metabolism below a standard which is lowered by five per cent, and the average metabolism ( $-7.1$  per cent) of the entire group is lower than such a standard. Tilt (11) found for a group of young college women in Florida that the average deviation from the Harris-Benedict prediction was  $-9.9$  per cent. She attributes this low value to the fact that the group studied by her is made up of southern women. Although our value is slightly higher than that reported by Tilt, there may not be sufficient difference to support a view that the basal metabolism differs for young women of the South and of the North. Our results do, however, show a wider range of variation,  $-20.0$  to  $+11.0$  per cent, than those reported by Tilt. Her results showed a variation which ranged from  $-18.5$  to  $+1.8$  per cent. Although the data presented in our investigation were obtained over a period of three years during the time from October to June, no difference in metabolism was observed with a change in season.

Since the group of women studied ingested on the average a fairly low protein intake and showed an average basal metabolic rate below the standard, it seemed possible that within the group an interrelationship might be apparent between protein intake and basal metabolism. The individuals of the group ingested daily quantities of protein which ranged from 0.5 gm. to 1.5 gms. per kilogram body weight and showed a range of deviation from normal basal metabolism of  $-20.0$  to  $+11.0$  per cent. In Table III summarized data are tabulated to show the basal metabolism averaged for groups ingesting different amounts of protein. The groups are, without doubt, too small to allow any definite conclusions. It seems apparent, however, from the data presented in Table III, that it is impossible to assign any definite relationship between habits of protein intake and basal metabolism. Although the small group whose daily intake was 0.5 gram protein per kilogram body weight showed the lowest basal metabolism, the group ingesting 1.2 grams protein per kilogram daily showed almost as low a basal metabolism. Also the average metabolic rate of the groups of individuals ingesting less than 0.9 grams protein is  $-6.5$  per cent, a value slightly higher than the average for the entire group and about the same as  $-6.8$  per cent, the average metabolism for the groups ingesting more



TABLE III  
BASAL METABOLISM AVERAGED FOR GROUPS INGESTING DIFFERENT AMOUNTS OF PROTEIN

Individuals in different groups	Protein intake per kilogram per twenty-four hours	Average deviation of actual basal metabolism from Harris-Benedict prediction
Number	Grams	Per cent
6	0.5	-10.8
8	0.6	-5.7
5	0.7	-6.8
9	0.8	-4.1
20	0.9	-7.6
14	1.0	-7.9
7	1.1	-6.9
6	1.2	-10.4
5	1.3	-4.9
4	1.4	-4.4
1	1.5	-4.0

than one gram protein per kilogram per day. Perhaps with a larger number of individuals and with data extending over a longer period of time, a more definite relationship could be demonstrated between protein intake and basal metabolism. Our present data appear to be in agreement with the recent findings of Wang and her associates (12) who could demonstrate no marked difference in the basal metabolic rate of subjects on different levels of protein intake. Kleitman (13), Deuel and his associates (14), and Wishart (15), on the other hand, observed a very definite difference in basal metabolic rate on different levels of protein intake. They found that the basal metabolic rate decreased as the protein intake fell and increased when the subject was put upon a high protein diet. In view of the findings of these investigators it seems possible that the somewhat lower protein intake of women may be in part responsible for the fact that their basal metabolism is usually lower than that of men.

#### SUMMARY

A study of the urinary nitrogen excretion, basal metabolism, and food intake of 85 college women was conducted for the purpose of determining the habits of protein consumption in women and the possible interrelationship between protein intake and basal metabolic rate.

The average daily protein intake of the group studied was 0.94 grams per kilogram body weight, a value slightly lower than the daily protein in-

gestion characteristic of male medical students as determined by Denis and Borgstrom, by Beard, and by Brooks.

The average twenty-four hour urinary nitrogen excretion for the group was 7.69 grams.

The average basal metabolism for the group was found to be lower than the standard. The deviation of the actual basal metabolism from the Harris-Benedict standard averaged  $-7.1$  per cent.

There appeared to be no definite interrelationship, within the group, between protein intake and basal metabolic rate. It is suggested that the lower protein intake of women may be in part responsible for the fact that their basal metabolic rate is usually lower than that of men.

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# VARIATIONS IN BLOOD SUGAR VALUES OF NORMAL AND VAGOTOMIZED DOGS FOLLOWING GLUCOSE ADMINISTRATION

By

J. P. QUIGLEY, W. R. HALLARAN, AND B. O. BARNES

*(From the Department of Physiology, Western Reserve University  
Medical School, Cleveland)*

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WE HAVE attempted to establish a method by which the administration of glucose would afford reliable information regarding the ability of an animal to burn or store (metabolize) sugar.

For this investigation we have employed the dog, an animal whose blood sugar level may remain relatively constant over a period of several hours. Quiet, gentle animals were selected and as a result of extensive training they would lie completely relaxed (although unanesthetized) for a period of hours on the beds provided. It was especially noted that they gave no evidence of psychic disturbance as the result of experimental manipulations, e.g., handling or the collection of blood samples. To reduce errors related to the absorption of previously administered food, the animals were in a fasting condition at the time of the experiment. The determinations were therefore made with the subjects in the basal condition. Errors related to variations in the rate of movement of material through the gut, and also the rate of absorption, were avoided by administering the glucose intravenously. We employed an amount of glucose which we determined by preliminary experiments was approximately the maximum which could be given under these conditions without losing glucose by way of the kidney. We made determinations of "true" blood sugar values; the method of Somogyi (1) was employed for these determinations.

Normal dogs, fasting 40 hours, received intravenously 0.25 gm. per kg. of glucose in the form of a 50 per cent solution. Approximately one minute was required for this injection. Blood samples were taken just preceding the injection and also 15, 45, 90, 180, 270 and 320 minutes after the injection.

A decrease in the demands made on the Islet cells of the pancreas, such as occurs during fasting, is believed by certain observers to reduce the readiness with which insulin is made available when glucose is subsequently administered. To test this theory, the experiment was repeated with dogs which had not been fed for 40 hours but to which 25 grams of

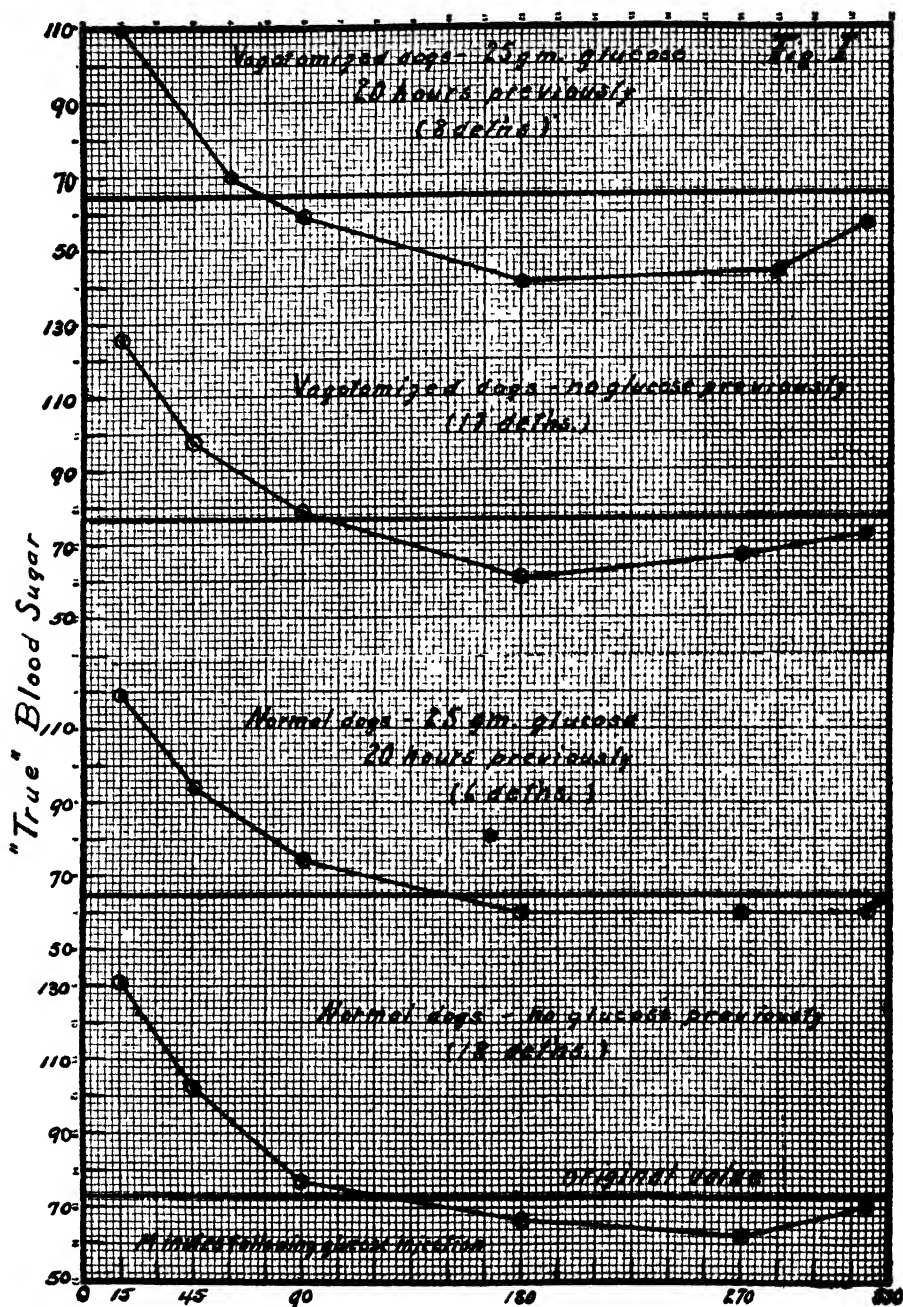


FIG. 1.—"True" blood sugar values following intravenous injection at 0 minutes of 0.25 gm. glucose per kg. Dogs fasting 40 hours.

glucose by stomach tube had been given 20 hours preceding the intravenous administration of the test solution of glucose.

Recent experiments of La Barre (2) have definitely shown that the vagi can be of great importance in determining the blood sugar level (or rate at which insulin is made available) following the administration of glucose. Since dogs with chronic bilateral vagotomy grossly show no indications of insulin insufficiency, we have employed the methods described above to compare such animals with normal dogs. We have not concerned ourselves with the acute changes which might follow section of the vagi (made just above the diaphragm) but have used animals 1 to 6 months after operation, animals in which adaptive changes would be well stabilized.

As was to have been anticipated, the blood sugar levels of animals apparently under the same experimental conditions showed some fluctuation. Certain animals gave figures consistently higher than others throughout the investigation and the curves obtained with a group of animals one day frequently were in closer agreement with each other than with those obtained from the same group on a subsequent day. The results of our investigation can most satisfactorily be presented in the form of graphs (Fig. 1).

Examination of these curves shows that the true blood sugar level of normal dogs fasting 40 hours averaged 73 mgm. It was practically the same in the vagotomized animal but decidedly lower in both groups of animals if fasting 40 hours and given 25 gm. of glucose 20 hours preceding the experiment. Fifteen minutes following the intravenous injection of glucose, the blood sugar level was lower in both groups of animals if dextrose was administered orally 20 hours previously. The blood sugar value regained the original level earlier in vagotomized animals and the fall below the original (degree of undershoot) was more marked than in the normal animal.

#### SUMMARY

A method is proposed by which a determination of the rate at which glucose is removed from the circulation following the administration of glucose would give information regarding the ability of the animal to metabolize sugar.

This method has been employed on normal and on double vagotomized dogs. Curves showing composite results are presented.

If glucose is administered 20 hours preceding the test, an increase in the ability to metabolize sugar is noted in vagotomized but not in normal animals.

Chronic vagotomy does not reduce the sensitivity of the mechanism which is stimulated by the administration of glucose; the mechanism rather appears to be slightly more irritable.

If these results are to be interpreted as indicating the rate of insulin secretion, they tend to show that hyperglycemia will lead to at least as great a secretion of insulin in the pancreas freed from central vagus control as in the normal Islet tissue.

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## Editorial Review\*

### EGG-YOLK PROTEINS

#### 1. INTRODUCTION

FOR many centuries, long before it was shown to contain not only readily-digestible protein of high biological importance but also iron, phosphorus, calcium and vitamins A, B, D, E and G, the egg has been recognized as one of the most valuable constituents of human diet. Particularly is this true of the yolk of egg, which, apart from its deficiency of carbohydrate and of the antiscorbutic factor, appears to contain all the food substances required for the normal development of young mammals. It is therefore all the more astonishing to realize, in spite of the large amount of time which, has during the past twenty years, been devoted to the study of proteins from the academic point of view, of egg production from the more technical standpoint, and of food constituents from both these aspects, that our knowledge of the chemical components of the egg-yolk has advanced so slowly.

We ourselves, engaged at present in a chemical investigation of the yolk proteins, venture to hope that it may prove useful for others interested to have before them a short review of the principal work which has already been done in this field. In this contribution we intend to limit ourselves, in the main, to a discussion of the chemistry of the yolk proteins of the unincubated egg. Work on the metabolism of the developing egg has already been adequately summarized up to 1924 by Needham (1) and more recent results of Calvery (2) have supplemented Needham's review. In order to keep the present article within reasonable and readable dimensions, we are excluding, as far as possible, the consideration of the other constituents of egg-yolk, namely, fats, lipins, sterols, pigments, vitamins, and salts.

In a brief review of this nature there must almost inevitably be errors of omission, and it may even be that some really important contributions have been overlooked. We hope that such omissions are few but would apologize in advance to any authors whose work we have unwittingly neglected.

#### 2. THE ISOLATION OF EGG-YOLK PROTEINS

(a) *Early work.* If its value is necessarily limited by the primitive conceptions of protein chemistry then extant, there is a certain historical interest in the work of the eighteenth and early

\* The authors of this review, Mr. T. H. Jukes, B.S.A. and Professor H. D. Kay, Ph.D., D. Sc., are, respectively, Bursar of the Ontario Research Foundation, and Professor of Biochemistry at the University of Toronto.



nineteenth century investigators on the yolk of egg. The earliest mention we have been able to find of protein in the yolk is that of Fourcroy (3) in 1782, who stated that yolk is chiefly "albumin," fat being the substance second in importance. Sixty years later, in 1841, Bence-Jones (4) coagulated egg yolk by heat and extracted fat from the coagulum with ether. On analysis of the residue its elementary composition agreed well with the usually accepted values for protein. Dumas and Cahours (5), a year later, named this protein "vitellin." Lehmann and Messerschmidt (6) in 1842 were the first to call attention to the unusual physical properties of this compound. They demonstrated that the precipitate which forms when egg yolk is mixed with water is easily soluble in either sodium or ammonium chloride solution, is reprecipitated from such solution by adding excess of water, and may be taken up again in salt solutions. Denis (7) made a further contribution to the method of purification of vitellin. After straining egg yolks through linen, he extracted them with ether until the extracts were color-free. The fat-free vitellin he found to be insoluble in water and saturated NaCl, but readily soluble in dilute acids, alkalies and 2 per cent and 10 per cent NaCl. It was rendered insoluble by long contact with water.

Apparently the first investigators to recognize the existence of two proteins in the egg yolk were Valenciennes and Frémy (8). They state that vitellin in the bird's egg yolk is always found associated with a certain quantity of albumin, hence, to prepare vitellin, they treated the egg yolk of the hen with cold water, the albumin remaining in solution while the vitellin was precipitated. The latter, washed with water, alcohol and ether, they regarded as a very pure protein.

An advance in the knowledge of the chemistry of vitellin was made by Hoppe-Seyler (9) who stated that vitellin was a lecithoprotein and showed that the lecithin could be split off by boiling alcohol (10).

Kossel (11) found that although the compounds guanine, hypoxanthine, and adenine could be separated from the "nucleins" of 15-day chick embryos, all of these compounds were absent from the yolk of unincubated eggs. He pointed out that the "nuclein" of milk (casein) and that of egg yolk (vitellin) contained no purine bases and were therefore essentially different from the "nuclein" (nucleoproteins) of the cell. In spite of this important observation, many years elapsed before a clear differentiation in nomenclature was made between nucleoproteins and phosphoproteins.

(b) *Work since 1899.* It is to be noted that the definition of vitellin as made originally by Dumas and Cahours had undergone modification. The name was now applied to the purified product prepared on the lines suggested by Denis' (7) work. Vitellin, as defined in this way, was first prepared in a reasonably pure form in 1899 by Osborne and Campbell (12). They mixed egg yolk with an equal volume of saturated NaCl, extracted the mixture repeatedly with ether, and dialyzed the fat-free residue. They obtained a series of fractions of various solubilities, which were found to contain from 15 to 30 per cent of "lecithin" on alcoholic extraction, the more soluble fractions containing the larger proportion of lecithin. It was found, however, that the insoluble residue obtained after a thorough extraction of any of these fractions with boiling alcohol had a very nearly constant composition (see Table I, substance 6).

In the same year Gross (13) extracted the ether-soluble materials from egg yolk, and filtered the resulting fat-free, whitish suspension through a Chamberland filter candle. Based partly on the investigation of the products obtained by this method of separation, and partly on an examination

TABLE I  
PERCENTAGE COMPOSITION OF THE PROTEINS OF THE YOLK OF THE HEN'S EGG

	Vitellin 1	Vitellin 2	Vitellin 3	Vitellin 4	Vitellin 5	Vitellin 6	Vitellin 7	Livetin 8	Vitellin 8
Carbon . . . . .					52.71	51.24			
Hydrogen . . . .					7.46	7.16			
Oxygen . . . . .					22.14	23.24			
Nitrogen . . . . .					16.64	16.38		15.35	
Sulphur . . . . .					1.05	1.04		1.80	0.6
Phosphorus . . . .						0.94		0.067	
Glycine . . . . .	1.10	0.00	trace	0.5					
Alanine . . . . .	+	0.75	0.16	0.5					
Valine . . . . .	2.40	1.87		1.5					
Leucine . . . . .	11.00	9.87	3.30	6.8					
Proline . . . . .	3.30	4.18	4.00	0.5					
Phenylalanine . . .	2.80	2.54	1.00	0.7					
Aspartic acid . . .	0.50	2.13	0.6	0.7					
Glutamic acid . . .	12.20	12.95	1.00	1.0					
Serine . . . . .	?	?		0.5					
Tyrosine . . . . .	1.60	3.37	0.40	2.0				5.2	5.0
Cystine . . . . .		?		—				3.9	1.4
Histidine . . . . .		1.90	trace	2.1			1.4		
Arginine . . . . .		7.46	1.20	1.0			7.6		
Lysine . . . . .		4.81	2.40	1.2			6.0		
Ammonia . . . . .		1.25		1.17					
Tryptophan . . . .		+						2.1	1.6

1. Abderhalden and Hunter (18).
2. Osborne and Jones (21)
3. Levene and Alsberg (20).
4. Hugounenq (19).
5. Osborne and Campbell (12).
6. Same as (5), but calculated to include the phosphorus.
7. Plimmer and Rosedale (22). Used the total egg-yolk protein
8. Kay and Marshall (15).

of the fractions obtained by ammonium sulfate precipitation, the conclusion was arrived at that a second protein, a globulin, was present in egg yolk. Credit for the recognition of the existence of this second protein belongs, as has just been stated, to Valenciennes and Frémy, and although Gross' work supported their results, a careful examination of his original paper leads us to the conclusion that at no time did he get a clear separation of the globulin and the vitellin. This was left for Plimmer (14) to achieve. Vitellin was prepared by diluting an ether-extracted solution of egg yolk in 10 per cent sodium chloride with a large volume of water, and filter-

ing off the precipitated protein. There was still a relatively large amount of protein nitrogen in the filtrate. To the filtrate, therefore, was added a little acetic acid, and the mixture boiled. The soluble protein was thus coagulated and could be filtered off and washed. Plimmer gave the name "livetin" (an anagram of vitellin) to this coagulum of which he obtained several grams. His yields varied from 10 gms. from 32 egg yolks to 30 gms. from 25 egg yolks. On analysis he found the phosphorus content to vary from 0.1 per cent to 0.65 per cent in 5 different specimens, being in all cases markedly lower than that of vitellin (1.0 per cent or above). He determined the nitrogen partition by the Hausmann method and found that the figures agreed fairly closely with those obtained for vitellin, but differed from those for ovalbumin. Livetin was higher in monoamino-N than vitellin, but lower in amide-N and diamino-N. He put forward the suggestion that the product he separated might be closely related to vitellin, might be, in fact, vitellin minus the phosphorus-containing fraction of the molecule. Although Plimmer brought evidence to show that his product was not ovalbumin, he did not demonstrate clearly that the proteins of the white did not contribute to his coagulum.

After Plimmer's work, nothing appears to have been done on the second protein of egg yolk until 1928, when Kay and Marshall (15) first obtained it in a fairly pure, undenatured form. After freeing the yolks completely from adherent proteins of the white, they were taken up in sodium chloride solution and extracted with ether. The fat-free protein solution was dialyzed against running water for a short time, and then poured into distilled water, until no more precipitate formed on further dilution. Most of the lecitho-vitellin was centrifuged off, and the remainder removed by filtration. The livetin was then precipitated from the filtrate by half saturation with ammonium sulfate and after repeating this precipitation twice more the product was extracted with alcohol-ether at  $-15^{\circ}$  by Hewitt's (87) method, which effectively removed traces of lipins remaining in the livetin. The resulting purified but uncoagulated protein had the properties of a pseudo-globulin, and on analysis was found to contain 15.1 to 15.35 per cent N, 1.8 per cent S, and 0.067 to 0.05 per cent P, the purer specimens having the smaller content of phosphorus. Amino-acid analysis by the methods of Folin and Looney (88) and Folin and Ciocalteu (89) gave 5.2 per cent tyrosine, 2.1 per cent tryptophane, 3.9 per cent cystine, whilst pure vitellin analyzed by the same methods at the same time gave 5.0 per cent tyrosine, 1.6 per cent tryptophane and only 1.4 per cent cystine, with a total sulfur content of 0.6 per cent. Plimmer's suggestion of the possi-

bility of a direct relationship between vitellin and livetin is not borne out by these results (see Table I).

Thus two definite proteins have been isolated from the yolk of the hen's egg, the first, a phosphoprotein, vitellin, which when combined with lecithin has globulin-like properties, the second, livetin, of very small phosphorus content and with the properties of a pseudoglobulin.

### 3. CHEMICAL CHARACTERISATION OF THE YOLK PROTEINS

Plimmer and Scott (17) showed that phosphoproteins such as casein and vitellin could be distinguished from nucleoproteins by the stability of the combined phosphorus of the latter to 1 per cent sodium hydroxide. The phosphorus of phosphoprotein was readily separated as inorganic phosphate by this treatment. Plimmer and Scott showed that the phosphorus-containing proteins of the roes of the sturgeon, herring, and mullet were similar to casein and vitellin in this regard.

Following the introduction by Fischer of the esterification method for determining amino-acid distribution, there was great activity for some years in the chemical investigation of proteins. The amino-acid content of vitellin was determined by Abderhalden and Hunter (18) who do not state their method of preparation; by Hugounenq (19) who used the heat-coagulated, total egg-yolk protein; by Levene and Alsberg (20) whose preparation, judged from the method given, must have been free from livetin; and by Osborne and Jones (21) who used a product which also must have been free from livetin. Their analytical results, and those of other workers on vitellin and livetin, are given in Table I.

Vitellin was found to contain 1.74 per cent tryptophan by May and Rose (23), 1.1 per cent tryptophan by Tillmans and Alt (24), 2.42 per cent tryptophan and 0.83 per cent cystine by Jones *et al.* (85). Ide (86) stated that dried egg yolk contained 2.46 per cent tryptophan. Livetin was found to contain from 14.82 to 15.35 per cent N, and 0.10 to 0.85 P by Plimmer (14). Other chemical properties of the yolk proteins are dealt with in later sections.

### 4. PHYSICAL PROPERTIES OF THE YOLK PROTEINS

*Vitellin.* Purified lecitho-vitellin is insoluble in water, and requires a rather greater concentration of salt to take it up into solution than does the typical globulin. The amount required varies with the length of time during which the protein has been in contact with water. After thorough extraction with ether of the yolk diluted with an equal volume of 8 or 10

per cent sodium chloride solution, and precipitation of the vitellin with water, there is still a considerable amount of "lecithin" loosely combined with the protein. This may constitute as much as 30 per cent or even more of the dry weight of the protein. At this stage the vitellin, or better, lecitho-vitellin, is easily soluble in 1 per cent salt solution or in dilute alkali, from which it can readily be precipitated by acidification. On keeping in contact with water, "lecithin" is slowly lost by the protein, which at the same time becomes less and less soluble in salt solution. After complete removal of the lipin, which requires treatment of the protein with hot ethyl or methyl alcohol, the vitellin becomes quite insoluble in water and almost insoluble in salt solutions or dilute alkali. It is, however, readily decomposed after suspending in dilute alkali, even at room temperature, both phosphoric acid and ammonia being liberated from organic combination.

Since vitellin becomes progressively less soluble in water the more it is purified, it is not surprising that few determinations of its physical constants have been attempted.

*Livetin.* Livetin, though completely precipitated by 50 per cent saturation with ammonium sulfate, is nevertheless soluble in water, or rather requires such small quantities of salt for solution that even on prolonged dialysis it remains for the most part unprecipitated. It may be said to have the properties of a pseudoglobulin. It is precipitated from aqueous solution by complete saturation with magnesium sulfate or sodium chloride, by three volumes of alcohol or acetone, by trichloroacetic, picric, tungstic, and sulfo-salicylic acids, by the salts of the heavy metals and by colloidal ferric hydroxide (15). Certain preliminary determinations of its physical constants have been made (15). (a) *Its isoelectric zone* in acetate buffers, is in the neighborhood of pH 4.8–5.0, and its titration curve shows a broad isoelectric zone in this region; (b) *The refractive index* for 1 per cent protein gives a mean value of 0.00190 at 20°C; (c) *The optical rotatory power* for the mercury green line determined on pure, alcohol-ether extracted specimens gives a mean value for the specific rotation at 20° as follows:  $[\alpha]_{5461}^{20} = -55.5^\circ$ . (d) *The minimum molecular weight*, on the assumption that there is only one atom of P in each molecule of protein, is in the neighborhood of 64,000.

*Relative quantities of vitellin and livetin* present in the yolk. A method for estimating the amount of livetin in the yolk of a single egg was developed by Kay and Marshall (15) on the assumption that all the non-vitellin protein in egg yolk is the same substance—livetin. (They considered at least 90 per cent of the non-vitellin protein to be livetin.) By this method

they found, both for hen's and duck's eggs, the ratio of vitellin to livetin to be between 3.3:1 and 4:1, i.e., between one-quarter and one-fifth of the yolk proteins of the hen's or duck's egg is livetin—the rest being vitellin. On this basis, and the figures given for sulphur content of yolk proteins in Table I, a short calculation will show that about one-half of the sulfur of the yolk is originally present as livetin. Whether their assumption (that at least 90 per cent of the non-vitellin protein in egg yolk is one protein—livetin) is entirely justified still remains to be seen. So far there is no definite evidence against it, except that yields of purified livetin are lower than might be expected on this assumption.

The ratio of vitellin to livetin in the egg yolk in the few cases in which it has been determined appears to be fairly constant. Whether this ratio varies in any way with the diet has not been ascertained. One possible explanation of the constancy of the ratio is that the two apparently distinct and certainly artificially-separable proteins are secreted or manufactured as one, and maintained in the natural state in the egg yolk as one large molecule which is decomposed at some stage during the process of fat extraction and "separation." If this is the case, the strength of the affinity between the two portions of the molecule must be small—considerably smaller, for example, than that between vitellin and lecithin in lecitho-vitellin. This question, however, has considerable interest in considering the mode of formation of the yolk, and may be of importance in yolk utilization by the growing embryo. Whether the two proteins are digested and absorbed by the embryo as one, or at least at the same rate, has not yet been determined.

There is, however, another reason why it is unlikely that the two proteins are in a state of loose chemical combination in the yolk. In some recent experiments (108) we have obtained evidence which suggests that livetin is very closely related to hen's serum globulin, and may, in fact, be identical with it. If these preliminary findings are confirmed, they would suggest a different origin<sup>1</sup> for the livetin and for the lecitho-vitellin of egg-

<sup>1</sup> A very short summary of some of the work on the minute anatomy of the formation of egg yolk and the yolk proteins may be given here.

The vitelline membrane enclosing the yolk is something more than a mere receptacle. Lecailon (102) found that in the bird *Turdus merula* the membrane was associated with an internal layer,  $3\mu$  in thickness, to which adhered fragments of the follicular epithelium and of the connective tissue of the theca from the ovary. Thing (103) observed in the ovarian egg of the turtle that prolongations of the epithelial cells of the yolk membrane traversed the zona pellucida and ended in knob-like enlargements in the yolk substance itself, presenting a very favorable arrangement for the conveyance of nutritive material from the epithelial area, in contact with the maternal capillaries, to the actively growing and extending yolk.

The laying down of yolk material, both protein and non-protein, within the growing egg yolk

yolk—the latter then being a specific protein constituent manufactured by the ovary, the former merely a translocated blood constituent. It is suggestive in this connection that Horvath (107) found recently that out of a number of laying hens whose blood constituents were determined, and all of which had a relatively high globulin to albumin ratio in the serum, the two best layers had by far the highest serum globulin figures (4.3 and 4.96 gms. per 100 cc. serum).

## 5. YOLK PROTEINS IN NON-AVIAN EGGS

It is of considerable interest to compare the results just described for the proteins of the yolks of avian eggs with those which have been obtained for the yolk proteins of the eggs of other classes of vertebrates.

Gobley (25), writing in 1850, states that Vauquelin in 1817 analyzed the eggs of the pike, and found much albumin, oily material, a gelatin-like substance, and mineral salts. Gobley himself used carp eggs and found them to contain 14 per cent of "paravitellin," a protein which he obtained by triturating the eggs with distilled water. He found iron present in the eggs in a fat-soluble form, and describes at some length his excellent and well-ordered researches upon the various fractions obtained from the eggs.

Valenciennes and Frémy (8) examined the eggs of many species of fish. From the eggs of cartilaginous fishes (principally rays and dogfishes) they isolated "ichtin" in which could be found no sulfur, while from the eggs of bony fishes they obtained "ichthulin," analogous to vitellin, containing 1.0 per cent S.

Walter (26), in 1891, prepared and purified ichthulin from carp eggs. His investigation is the only one we have been able to discover in which the iron content of this protein is recorded. Two determinations, by different methods, gave him 0.09 per cent and 0.117 per cent—rather high values. Ten years later Levene (27) extracted ichthulin from cod-roe by repeated solution in 5 per cent  $\text{NH}_4\text{Cl}$  and precipitation by dilution. He noted that the substance became orange-yellow after extraction with absolute alcohol and ether.

Hugounenq (28) isolated a water-insoluble protein from the eggs of the herring, which he named "clupeovitin." He regarded it as a complex, feebly acid protein very similar to the vitellin of birds' eggs. It was readily soluble in dilute alkalis and in dilute  $\text{HCl}$ , and contained small quantities of P and Fe. A similar protein was obtained by Galimard (29) from the eggs of *Rana esculenta*, and named "ranovitin." Ichthulin from the eggs of *Squalus acanthias* was prepared by

is a process the understanding of which is of first importance if the biochemical relationship of the hen to the egg is to be made clear. What concerns us more immediately at present is the production of the yolk protein by the hen, but this cannot, as yet, be disentangled from the production of the other yolk constituents. That this process is concerned with the so-called "vitelline body of Balbiani," which is to be found in the cytoplasm of the young oövules of a wide variety of species, seems to be the opinion of several cytologists. Henneguy (104) in 1893 described this structure as a finely granular mass situated not far from the periphery of the egg, with a small, more deeply staining body centrally within it. D'Hollander (105) studied the cells in the ovary of the embryo chick, and showed that Balbiani's body is already present in the oöcyte. It is closely related to the attraction sphere—the dormant centrosome. He considered that the body of Balbiani, surrounded by its "vitellogenic bed" played a major part, especially in birds, in the formation of the nutritive yolk. Lams (106) working with the frog *Rana temporaria* stated that during the growth of the oöcyte, its cytoplasm is increased in quantity by "deutoplasmic bodies of a mitochondrial nature" elaborated under the control of the sphere of attraction, which remains in the oöcyte as the vitelline body of Balbiani.

Alsberg and Clark (30). After ether and hot alcohol extraction they found, rather surprisingly, that phosphorus was absent, and that only slight traces of iron could be detected. Attempts to produce hematogen (see p.91) from it by the usual methods successful with vitellin, yielded very small amounts of a substance which contained no P and but slightly more Fe than the original material.

Reinvestigation of this dogfish egg-yolk protein has been made recently by Needham (31). He was able to show that, like avian egg-yolk protein, it could be separated into two fractions, the ichthulin fraction, analogous to vitellin, and another fraction, similar in properties to livetin, which he called "thuichthin." The phosphorus content of the former protein after thorough extraction with boiling alcohol he found to be 0.62 per cent, which is not in agreement with the findings of Alsberg and Clark, whilst "thuichthin" contained only 0.039 per cent of phosphorus.

Fauré-Frémiet and Garrault (36) found the ovarian egg of the carp (*Cyprinus carpio*) to contain 25.7 per cent of its total weight as protein. They extracted it with boiling alcohol and estimated its nitrogen and phosphorus content. They also (37) report similar results with a protein from the eggs of the trout (*Trutta fario*).

Steudel and co-workers (34) give figures for the basic-N distribution of herring ichthulin when analyzed according to the method of Kossel and Kutscher (90).

McClendon (32) found the yolk platelets of the egg of *Rana pipiens* to contain 94 per cent of a protein which he named "batrachiolin." Its composition and precipitation reactions indicated its relationship to vitellin and ichthulin. Later (33) he found that the protein dissolved slowly in salt solutions, but was more readily soluble in alkalies. It was completely precipitated by half saturation with ammonium sulfate. The black pigment of the eggs he found to contain 0.483 per cent P, 0.83 per cent S, and 10.9 per cent N; he states, however, that the phosphorus content of the pigment was apparently due to contamination with batrachiolin.

Fauré-Frémiet and de Stréel (35) separated "vitellin flakes" from the eggs of *Rana temporaria* by centrifuging the crushed eggs. They were able to separate partially two protein fractions by treatment with N/10 NaOH, the soluble part (37%) being a nitrogen compound rich in P; the residue was a nitrogen compound containing little or no P but some S, and very resistant to reagents.

Fauré-Frémiet (38) separated "vitelline globules" from the eggs of a marine worm, *Sabellaria alveolata*, and found them to contain two nitrogenous fractions, one apparently an albuminoid, the other soluble in dilute alkali and containing 0.51 per cent of P.

Komori (57) prepared a protein from the spawn of the gastropod *Hemifus tuba* (Gmel) by extracting with alcohol and ether the coagulum obtained by boiling the spawn in dilute HCl. He quotes for the amino-acid content of this "crude vitellin," glycocoll 0, alanine 0.71 per cent, valine 0.27 per cent, leucine 10.29 per cent, isoleucine 0, proline 1.10 per cent, phenylalanine 0.22 per cent, aspartic acid 1.60 per cent, glutamic acid 0, serine 0, tyrosine 0.80 per cent, arginine 3.73 per cent, histidine 0, lysine 0.86 per cent, tryptophan 1.49 per cent.

Tomita (39) found the eggs of the sea turtle (*Chelonia cavana*) to contain 39 per cent of white, of which only 1.5 per cent was solid matter, and 55 per cent yolk. Thirty-seven per cent of the yolk was solid matter, containing 10.7 per cent N and 4.3 per cent ash.

The analytical findings of several of the above mentioned investigators are given in Table II.

## 6. WHITE AND YELLOW YOLK

The question of the homogeneity or otherwise of the egg yolk has not yet been discussed. It is not difficult to show, in a hard-boiled egg, that the yolk is made up of alternate layers of white and yellow yolk. That the difference between these layers is more deep seated than one of mere pigmentation has been shown by the work of Riddle, and of Riddle and Spohr.



TABLE II  
PERCENTAGE COMPOSITION OF THE PROTEINS OF NON-AVIAN EGG YOLK

	Paravitellin 1	Ichthulin 2	Ichthin 3	Ichthulin 4	Clupeovin 5	Ranovin 6	Ichthulin 7	Ichthulin 8	Ichthulin 9	Thulichthin 9	Ichthulin 10
Carbon	52.6	53.52	51.0	52.5	53.68	53.61		50.49	48.80	45.60	52.44
Hydrogen	7.74	7.70	6.7	8.0	7.38	7.79		7.16	7.58	7.39	7.45
Oxygen	25.24	22.19	25.4	22.7	23.90	24.27					22.58
Nitrogen	15.15	15.64	15.0	15.2	14.64	15.32		16.76	14.75	12.14	15.96
Sulphur	0.90	0.41	—	1.0	0.40	trace		0.91	1.17	1.96	0.92
Phosphorus	0.37	0.43	1.9	0.6	trace			0	0.62	0.039	0.65
Iron	+	0.10			trace						
Leucine					21.2	13.02					
Tyrosine					1.0	1.03					
Histidine					0.4	1.14	2.5*				
Arginine					2.7	1.06	14.5*				
Lysine					2.0	0.29	10.1*				
Ammonia							1.8*				
Humin-N.							I-6.8*				
							II-1.7*				

1. Gobley (25) From carp eggs.
2. Walter (26) From carp eggs.
3. Valenciennes and Frémy (8) From cartilaginous fishes.
4. Valenciennes and Frémy (8) From bony fishes.
5. Hugounenq (28) From the herring.
6. Galimard (29) Yolk protein of frog's eggs.
7. Steudel *et al.* (34) From herring roe.
8. Alsberg and Clark (30) From the eggs of the spiny dogfish.
9. Needham (31) From the eggs of the dogfish.
10. Levene (27) From cod-roes.

\* Figures are for basic-N distribution. Non-basic-N=61.8%. Additional figures include McClendon's (32) analysis of "Batrachiolin" from the eggs of *Rana pipiens*; N=15.14%, S=1.32%, P=1.21%; and Fauré-Frémiet and Garrault (36) for carp ichthulin; N=14.16%, P=0.573% and trout ichthulin (37), N=14.28%, P=0.57%.

Riddle stated (45) in 1911, that these alternate layers are due to a daily rhythm of nutrition, probably associated with rhythmic changes in the blood pressure. The layering was quantitatively most significant in the last 5 to 8 days before extrusion, during which time more than 99 per cent of the yolk in the ovum was manufactured, months of slow growth being followed by a rapid increase in size after the diameter of 6 mm. had been reached. This sudden change might be due to an increase in permeability of the follicular cells. He was of the opinion that yolk formation, like the

majority of other types of tissue anabolism, was essentially reversible in nature, being in fact analogous to a reversible chemical change catalyzed by enzymes. He withheld opinion with regard to the production and possible reabsorption of the yolk *proteins*, but believed that fats were split before passing into the yolk. Arguing from the rapid growth in the later stages, he considered that in birds the yolk was not exclusively derived from the nucleus or from within the follicular cells. In Riddle's opinion the two chief factors in yolk formation were: (a) reversible action of enzymes, (b) partition coefficients of solubility in the yolk and in the maternal blood of the several yolk constituents. Spohr and Riddle (46) analysed the white and yellow yolk, and found that there was a very marked difference in composition. Whilst white yolk, formed at night, had a composition of water 86.7 per cent, phosphatides 1.13 per cent, neutral fat 2.39 per cent, extractives 0.40 per cent, ash 0.62 per cent, and protein 4.6 per cent, the yellow yolk, deposited in the day time had water 45.5 per cent, phosphatides 11.15 per cent, neutral fat 25.2 per cent, extractives 0.36 per cent, ash 0.44 per cent, and protein as high as 15.04 per cent. (Egg white contains 10–11 per cent of protein.) They pointed out that it is the *white* yolk which is at all stages in most intimate contact with the egg nucleus, the blastoderm, and the embryo. The percentage composition of the white yolk is nearer to that of living, growing tissues than is that of the yellow yolk.

Since the proportion of white yolk in the whole yolk of the hen's egg is probably less than 5 per cent (Riddle) the total contribution of the white yolk to the yolk proteins is small.

#### 7. IRON AND THE YOLK PROTEINS—"HAEMATOGEN"

In the closed system of the egg, all the iron necessary for the synthesis of the haemoglobin in the blood of the embryo chick must obviously be present within the shell when the egg is laid. Actually, at the beginning of incubation, nearly all this iron is present in the form of an organic complex, not soluble in fat solvents, associated with the proteins of the yolk. From an average of 38 samples it has been found by us that the total yolk protein after completely extracting all the lipins with boiling alcohol contains 0.045 per cent of iron.

Peptic digestion of the yolk protein was found by Miescher (50) in 1870 to split off an insoluble product which Bunge (51) later named "haemato-gen." This material was investigated in more detail by Hugounenq and Morel (52, 53). The last authors found it to contain 0.455 per cent Fe and 8.7 per cent P, whilst Bunge had reported the smaller iron and phosphorus content of 0.29 per cent Fe and 5.19 per cent P. Hugounenq and Morel

(53, 54) believed that haematogen was a relatively complex substance, a type of conjugated protein in which an iron-containing pigment which they isolated and named "haematovin" was the prosthetic group, combined with a protein derivative. The combination was rather similar to that between globin and haematin to form haemoglobin. Haematovin, obtained as a brown powder by boiling haematogen with 30 per cent sulphuric acid, contained 2.6 per cent of iron and 0.1 per cent of phosphorus. It was insoluble in organic solvents but soluble in dilute alkali. Other than its content of organically combined iron, it had none of the characteristic properties of haematin, though certain oxidation products of haematin were obtained with an elementary composition similar to haematovin. Hugounenq and Morel regarded haematogen as a kind of undifferentiated, reserve haemoglobin and haematovin as "an incompletely differentiated and embryonic state of haematin." Some support for this belief lies in the more recently discovered fact that coincident with the sudden decrease in the vitellin of the yolk (Plimmer and Scott, 17) there is a corresponding rapid increase in the haemoglobin content of the chick about the 14th day (Sendju, 40).

Posternak (55) has recently prepared an enzyme-resistant, iron-containing material from the protein residue left after ether extraction of egg yolk. A fraction rich in iron (3.6%) and phosphorus (12.4%) was obtained after peptic and tryptic digestion of this residue.

It seems probable that haematogen is not a definite compound. It is likely that the iron-containing and the phosphorus-containing portions of the vitellin molecule are closely associated with one another, and are present in a portion of the molecule relatively resistant to enzyme hydrolysis. Depending on the extent to which the less stable portion of the protein molecule is hydrolyzed away, compounds containing more or less phosphorus and iron will be obtained in the "haematogen" separated. The ratio of Fe to P will probably be fairly constant (although the absolute values for the two elements will vary), provided the method of preparation is not dissimilar. This is exemplified by the analyses of Hugounenq and Morel given above (Fe:P = 1:19) compared with those of Bunge (Fe:P = 1:18).

Walter (56) prepared a compound from carp ichthulin in a similar way to that used by Bunge to prepare haematogen from vitellin. Walter's compound contained 0.25 per cent Fe and 2.6 per cent P.

Hugounenq and Morel (53), as would not be unexpected after Kossel's (11) demonstration that recognisable quantities of purines were not present in hen's eggs, found no xanthine bases in haematogen. Reducing sugar was also absent. It is worthy of mention here that, using the refined Krü-

ger-Schmid (97) technic, Mendel and Leavenworth (71) have since shown that a very small amount of purine N (0.0016 gm. N per egg) is present in fresh hens' eggs.

*Effect of diet upon the Fe content of the egg.* There was disagreement among earlier workers as to whether the iron content of egg yolk might be increased by feeding diets rich in iron. Hoffman (44) and Schmidt (43) claimed positive results, while Hartung (42) was unable to show such an effect. Recently Elvehjem, Kemmerer, Hart, and Halpin (41) state that the quantity of iron in egg yolk cannot be increased by feeding 50 mgm. Fe daily to the laying hen. (See also p. 98.)

*Vitellinic acid.* A compound with a similar Fe to P ratio was isolated by Levene and Alsberg (70) from vitellin by a method which did not entail an enzymic hydrolysis. Vitellin was suspended in water, and treated with strong ammonium hydroxide solution, which was allowed to remain in contact with the protein for two hours. The ammonia was then slowly neutralized with acetic acid, and excess of picric acid was added, and the reaction mixture filtered. On adding alcohol to the filtrate, a precipitate was obtained, purified as far as possible by repeated solution and reprecipitation, and finally extracted with boiling alcohol and ether. The product contained 0.57 per cent Fe and between 9.7 and 10.0 per cent P. Sulphur (0.3 per cent) was present. The Fe:P ratio (1:17 to 1:17.5) is quite close to that reported for haematogen. Levene and Alsberg's substance, which they call vitellinic acid, gave relatively large quantities of "melanin" on boiling with hydrochloric acid, and contained arginine and histidine. As might be expected from the relative lability of the phosphorus of phosphoproteins to alkali, this substance lost most of its phosphoric acid on warming with 2 per cent sodium carbonate. They showed that the iron was present in a relatively firm state of combination—i.e., organically bound.<sup>2</sup>

Assuming a 10 per cent P content for vitellinic acid, the maximum possible yield from vitellin (with a P content of about 1 per cent) is some 10 per cent of the weight of the vitellin. Kay and Marshall (95) in several experiments obtained yields of only 3.5 per cent or less of the weight of the vitellin. It would appear therefore that only a fraction of the vitellin phosphorus occurs in the vitellinic acid portion of the protein molecule. The approximate equivalent weight of vitellinic acid (2 specimens), using standard soda and thymol-phthalein, was found to be 203 and 216. It was found to contain, at the most, only traces of sulfur, apparently no tyrosine, but about 1 per cent of tryptophan (vitellin contains about 0.6 per cent

<sup>2</sup> Levene (27) prepared a very similar compound, "ichthulinic acid" from fish eggs.

of sulfur, 5 per cent of tyrosine, and 1.6 per cent of tryptophan (15)). Unlike the phosphopeptone derived from caseinogen, vitellinic acid appears not to lose its phosphoric acid on treatment with kidney phosphatase. It is therefore probably not a simple phosphoric ester.

The proteins of the hen's egg, like those of the milk in mammals, are secreted relatively rapidly by a comparatively small organ of the body. Ninety-nine per cent of the 3 grams or so of protein in the egg yolk is secreted during the last five to eight days before laying. Evidently one or both of two sources is possible for these proteins; they may be produced in the gland by the reversible action of proteolytic enzymes by direct combination of the amino acids circulating in the maternal blood plasma, or they may be derived (possibly by a process which does not entail complete breakdown to amino acid and resynthesis, but some far less drastic change in the protein molecule) from the circulating proteins of the blood plasma. Rimington (91) has pointed out that in their distribution of nitrogen as determined by Crowther and Raistrick (92), casein and lactoglobulin (serum globulin) are not remotely dissimilar, and has himself shown that artificially phosphorized serum globulin has several similarities to casein.

Whether or no any such considerations may be applied to the secretion of the egg-yolk proteins, i.e., whether they are synthesized mainly from the circulating amino acids of the mother's blood, or whether they are more or less simple modifications of the plasma proteins, we have as yet little more than surmise to guide us. It is perhaps unwise, even in surmise, to limit the possible alternative sources of the egg-yolk protein to the two just mentioned. In view of the high iron content of the egg-yolk proteins, and of the belief of some investigators that they contain an iron-rich nucleus which, with relatively few chemical changes is transformed directly into haemoglobin in the blood of the embryo chick (see p. 92 under haematogen), it is not impossible that the maternal red cells may be called upon for a contribution not merely of the iron, but of the iron attached to a fragment of fairly large molecular size, derived from haemoglobin by processes which involve only limited hydrolytic scission.

## 8. PHOSPHORUS AND THE EGG-YOLK PROTEINS

A number of empirical facts have already been quoted with regard to the relationship of phosphorus to the egg-yolk proteins. Lecitho-vitellin, prepared in the usual way, contains organically combined phosphoric acid in two forms, one detachable from the protein molecule by boiling with ethyl and other alcohols, the other form more firmly united to the protein, and probably present in the iron-containing portion of the protein molecule.

Like the organically-bound phosphoric acid of casein, but unlike that of lecithin, vitellin phosphorus is readily liberated as inorganic phosphate by warming with alkali. Bayliss and Plimmer (72) suggested that the lecithin portion, detachable from lecitho-vitellin by alcohol, contains one-half of the total phosphorus of the protein. This finding is probably a fortuitous one, since the lecithin content of lecitho-vitellin varies widely with the method of preparation, and is probably far from a constant quantity. As already stated, Plimmer and Scott (17) showed that nuclein phosphorus was absent from vitellin. They later (73) give the figures shown in Table III for P distribution in the unincubated hen's egg.

TABLE III  
PHOSPHORUS DISTRIBUTION IN UNINCUBATED EGGS. PERCENTAGE OF TOTAL PHOSPHORUS

	Hen's egg (Plimmer & Scott (73))	Frog's egg (Plimmer & Kaya (74))
(a) Inorganic P. . . . .	trace	0
(b) Water-soluble organic P. . . . .	6.2	4.3
(c) Ether-soluble P. . . . .	64.8	26.2
(d) Phosphoprotein P. . . . .	27.1	61.9
(e) Nuclein-like P. . . . .	1.9	—
(f) Protein P (Phosphoprotein + nucleoprotein) . . . . .	—	69.5

Unfortunately, in Plimmer and Scott's figures for the hen's egg, (e) is obtained by subtracting  $a+b+c+d$  from 100, so that its value as evidence of the presence of nucleoprotein or nucleic acid P is not outstanding. It would appear almost certain that any nucleic acid of fresh egg yolk is confined to the minute amount in the germ spot. Chapin and Powick (98) found that only 3.7 per cent of the total P of whole egg was present as inorganic phosphate. The remaining 96 per cent which is organically combined is almost entirely present in the yolk in the form of vitellin (some 16 per cent of the yolk) and phospholipins (about 11 per cent of the yolk). König (99) quotes Goble as stating that 1.2 per cent of the yolk consists of glycerophosphoric acid, although Kay (100) found no substrate for phosphatase in fresh eggs. It is by no means difficult to obtain free glycerophosphoric acid, during the usual processes employed for precipitating proteins and lipins from the yolk, from its precursor the yolk lipin (100a), and the presence of this acid in the unincubated egg must be considered, at present, as being very doubtful. Pine (75) found the acid-soluble portion of the phosphorus of the egg to increase during storage.

Little is known with regard to the mode of linkage of the organically

combined phosphorus of lecithin-free vitellin with the rest of the molecule. It is, however, almost certainly present as an ester of phosphoric acid. Rimington (93) analyzed the phosphorus-rich peptone prepared by Rimington and Kay (94) from casein, and found that the phosphorus was associated with hydroxyamino acids,  $\beta$ -hydroxy-glutamic, hydroxyaminobutyric acids and probably serine being present in the peptone. It was concluded that the phosphoric acid was probably combined with hydroxy-groups of the hydroxy-amino acids. One-third of the phosphorus of the peptone appeared to be rather differently attached to the amino acids; the evidence suggested the possibility that in this fraction *two* of the hydroxyl groups of the phosphoric acid might be esterified. It is not unlikely that some similar form of combination is present in vitellin. In fact Posternak (76) has claimed the presence of a phosphorus-containing nucleus in vitellin, in which several serine-phosphoric acid complexes are combined.

As already noted, pure specimens of livetin contain very little phosphorus, but even after careful purification, 0.05 per cent P is so obstinately retained that it is probably a true component part of the protein molecule.

Egg yolk is similar to milk, another rapidly produced physiological secretion designed for the nutrition of the very young animal, in its high content of phosphoprotein and its very low nucleoprotein and inorganic phosphate content. In fact, outside these two sources, phosphoproteins hardly occur at all in nature. Like milk, egg yolk contains a fairly high proportion of fat, but it is dissimilar from milk in its high lecithin and iron, and very low carbohydrate content.

The fate of the phosphorus of the yolk during incubation was examined by Plimmer and Scott (73). Their results suggested that the phosphorus of the bone of the embryo chick came largely from the lipin of the yolk, whilst the vitellin P gave rise partly to additional inorganic phosphate and partly to the nucleoprotein of the tissues. As we do not purpose extending this review to cover the process of incubation, these interesting results will not be discussed here. It would, however, seem desirable that the work of Plimmer on the metabolism of phosphorus in the developing egg should be extended, using modern methods of phosphorus determination.

Analyses of the elementary composition of the egg and the yolk, and of the ash derived from these, are to be found in König's (99) book published in 1904. Some of these figures, which are to be found in several more recent text books, and are quoted as authoritative, are taken from Gobley's work (25a) published in 1846. Again it would seem high time that modern methods of analysis be applied to such an important dietary constituent as the egg.

## 9. NUTRITIONAL VALUE OF EGG-YOLK PROTEINS

The proteins of the hen's egg must contain all the essential amino acids required for the growth of the young bird from an embryo of a few dozen cells to the complete chick ready for hatching. That is, the amino acids tryptophan, lysine, cystine, and histidine *inter alia* must be present in adequate quantities. It is in the highest degree likely that proteins biologically valuable for the growth of one species of vertebrate will be biologically valuable for another species, and this has been shown to be the case for egg proteins. Osborne and Mendel in 1912, 1913 (47) demonstrated that good growth is obtainable in rats with ovovitellin as the sole source of protein. Although their experiments were complicated somewhat by the fact that at that time very little was known with regard to the accessory food factors, their growth curves would suggest that vitellin was, in fact, superior for growth of rats to most of the proteins tested. De Sanctis (48) pointed out the value of egg yolk as a food for athreptic infants, and generally for infants who, though receiving the normal maximum diet, refused to grow. Smith and Chick (101) also bear testimony to the value of egg yolk in the diet of lactating rats whose litters were not thriving. This work was confirmed and extended by Clayton (48a) who found egg yolk superior to other food proteins for reproduction and lactation in the rat. In both these cases the situation is almost certainly controlled in the main by the vitamin content of the yolk, and probably hardly at all by the specific nutritional value of the egg-yolk proteins. To deal with the vitamin content of the egg yolk in this review would unfortunately lead us too far afield.

Mitchell and Carman (49) found in 1924 and 1926 that the protein of *whole* cooked egg had the highest biological value (94 per cent) of any they obtained from a food, surpassing even that of milk (85 per cent), the next highest, and of egg white (83 per cent). The relative figures for egg white and whole egg would suggest that the egg-yolk protein would be found to be even more valuable than that of the whole egg. Although we still lack adequate figures for the amino-acid composition of the egg-yolk proteins, certain at least of the biologically important amino acids (e.g., tryptophane and cystine) are known to be present in relatively large amounts in these proteins (see pp. 83 and 85).

## 10. THE EFFECT OF DIET ON THE COMPOSITION OF THE HEN'S EGG YOLK

That the composition of the egg yolk may be influenced by the diet of the fowl has been shown by Bethke, Kennard, and Sassaman (79) whose evidence indicated clearly that the vitamin A and vitamin D content of



egg yolk is largely, if not entirely, determined by the amount of these substances present in the diet. Whether the amino acid make-up of the protein is capable of influence in the same way was investigated by Pollard and Carr (77) and Gerber and Carr (78) and, from a different angle, by McFarlane, Fulmer, and Jukes (63). Pollard and Carr fed pigeons on diets restricted to a single seed grain for proteins and vitamins. On analysis, the resulting eggs showed a wide difference from one grain group to another in their total nitrogen. The distribution of nitrogen in six fractions also varied greatly in eggs from different diets. Only the eggs which gave a high melanin yield were hatchable. The formation of melanin is known to be dependent on the presence of tryptophan in the protein molecule. Gerber and Carr found that the mono-amino nitrogen was higher in the eggs obtained after the feeding of hemp, soy-bean or wheat, whilst the eggs obtained after feeding kafir showed a higher diamino-nitrogen content. They also obtained some evidence from anaphylactic reactions with guinea-pigs and rats that the egg proteins obtained from pigeons on the different diets differed immunologically. The rate of development of the embryo was also influenced markedly by the quality of the protein in the diet of the mother birds.

McFarlane, Fulmer, and Jukes took up the finding of Graham and Smith (96) that successful hatching of a fertile egg depended to a considerable extent on the nature of the protein supplement given to the hens in addition to the basal vegetable ration, and made a careful chemical investigation of the eggs produced by hens on different protein diets. They determined the amount of the essential amino acids tyrosine, tryptophane and cystine in both egg-white and mixed egg-yolk proteins, and found no significant difference in the content of any of these three amino acids in either the egg-white or the egg-yolk protein from one dietary group to another. In particular the percentage of tryptophan determined by three methods of analysis showed no significant variation. Nor did iron content of the yolk protein vary very materially from one group to another. There was, in fact, no clear evidence that the diet of the hen had any influence on the amino-acid or iron content of the egg-yolk (or egg-white) proteins.

These results are not, perhaps, surprising. The constancy of composition of the proteins of tissues and of secretion, from one individual in a species to another, whatever the diet, is a finding which has not yet been contradicted by any experimental evidence with the possible exception of that of Pollard and Carr just quoted. It is frankly difficult, however, to find direct experimental basis for the opinion, which would probably be held by most authorities on what appear to be *a priori* grounds, that any serious disturb-

ance in the content of essential amino acids in the diet of laying hens would lead first to a slackening of egg production, without any observable change in the composition of the individual proteins of the yolk (although their relative quantities might possibly vary), and would later result in complete cessation of egg production. It would seem that further work on this important aspect of nutrition not only with regard to egg protein, but with regard to other proteins resulting from anabolic and secretory processes, is urgently required, from the practical no less than from the more academic standpoint.

T. H. JUKES  
H. D. KAY

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# A DOCTOR OF THE 1870's AND 80's

*By*

WILLIAM ALLEN PUSEY

*Sometime President of the American Medical Association and of  
the American Dermatological Association*

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THE EFFECT OF GRAPES AND GRAPE PRODUCTS  
ON URINARY ACIDITY

BY LAWRENCE G. SAYWELL

*(From the Fruit Products Laboratory, University of  
California, College of Agriculture, Berkeley)*

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## INTRODUCTION

DURING the course of a study in this laboratory of the composition of California grapes and grape products, it was observed in all cases that the ash was decidedly alkaline, and it seemed likely that they might be of value in the diet for the purpose of maintaining the alkaline reserve. Since many fruit acids are largely oxidized to carbon dioxide and water in the body, the ash constituents rather than the organic acids are the chief factors affecting the acid-base balance of the body. However, some fruits have been shown to increase the acidity of the urine even though the ash was alkaline. Consequently, it has seemed desirable to secure additional information upon the effect of grapes and grape products upon the acidity of the urine.

The alkalinity or acidity of the ash of many food materials has been reported by Sherman and Gettler (15) who give a high alkalinity for the ash of raisins. Numerous published analyses of fresh grapes (2, 3, 14, 15) have indicated that a basic ash is characteristic of all varieties. Richert (13) has published results of his determinations of the alkalinity of the ash of several California (*Vinifera*) grape juice concentrates which indicate that the alkalinity is several times that of fresh grapes. Hartman and Tolman (8) have found the alkalinity of the ash of Concord or so-called Eastern grape juice to be relatively high.

Blatherwick (4) found that the addition of raisins to an otherwise acid diet resulted in decreased acidity of the urine. However, he did not state the source of the raisins nor did he report a study of fresh grapes and grape products. Therefore, it appeared desirable to ascertain whether the property of reducing the urinary acidity is characteristic of California varieties of grapes and their products. Pickens and Hetler (12) in testing the effect of a commercial Concord grape juice, found that "no marked change oc-

curred in the acidity of the urine, even when large amounts of grape juice were ingested." Their results and those of Blatherwick will be referred to later in this paper and a suggested explanation given for the results obtained by Pickens and Hetler.

Various periods have been employed for determining the body reaction equilibria both with a basal diet and with a basal diet plus the food material being studied. Blatherwick (4) used a five-day period for determining the effect of the basal diet. With regard to the influence of an added food on the composition of the urine, he stated that "the body reacts quickly to the change, and the new equilibria will generally have been established by the second day. Somewhat less than a week is sufficient to obtain conclusive data." Two subjects were used by them for study of the influence of each of several foods. Blatherwick and Long (5) in studying the effect of drinking large amounts of strained orange juice employed a four-day basal diet period followed immediately by a four-day period with daily increasing amounts of the juice added to the basal ration. Two subjects also were employed in these studies. In a corresponding study of the effect of sour milk, they employed two five-day periods with two subjects. Chaney and Blunt (6) studied the effect of orange juice on two growing children and in one case employed a preliminary period of three days during which no analyses were made. In the second case, the preliminary period was of five-days' duration with no analyses made. In both cases analyses were made during the following three days of basal diet, then after a delay of a day or two with basal plus orange juice, analyses were made for three days' continuation of the use of orange juice. Pickens and Hetler (12) studied the effect of grape juice on nitrogen retention and urinary acidity. Their experiments were divided into two periods. The first five days were for adjustment and usually no collections of urines or feces were made. A three- or four-day period followed in which daily urines were collected under toluene and analyzed for nitrogen. They reported their results as averages for the periods during which analyses were made. No data are given for any of the daily analyses. Two subjects were employed in each case. The results of Blatherwick (4), Blatherwick and Long (5), and Chaney and Blunt (6) indicate that equilibrium is obtained within five days on the basal diet. In like manner, it is shown that equilibrium was again attained within two or three days after the addition of the food to be studied.

#### EXPERIMENTAL

In this investigation the effect of fresh grapes and of several grape products on the reaction, ammonia content, total acidity and organic acid

content of the urine was determined. All samples were of known history. The fresh grapes were taken from a shipment of well ripened commercial Malaga grapes which appeared to be of uniform maturity and representative of the variety. The edible portion contained 23.6 per cent total solids. The natural grape juice was prepared by mixing one part of Malaga, one part of Muscat and one part of Petit Sirah freshly pressed grape juices, all containing 21 per cent total solids. The individual juices were portions taken from fifty to one hundred gallon quantities of juice prepared from mature, sound fruit. The detartrated grape juice was prepared with the same proportions of the individual juices but was detartrated by freezing (10). The natural grape juice concentration was prepared by mixing equal portions of the concentrated juices of the three varieties of grapes. The concentrate contained 72.3 per cent total solids. The concentrate-tartrate mixture consisted of one part concentrate and four parts of the tartrate precipitated from the juice by freezing. The Thompson seedless, and Muscat seeded raisins were from fifty-pound lots of the standard, commercially packed, dried fruit.

The basal diet was similar to that used by Chaney and Blunt (6), being as follows: whole milk, 275 gms.; oatmeal, 20 gms.; sugar, 10 gms.; white bread, 310 gms.; butter, 45 gms.; potato, 200 gms.; rice, 55 gms.; raw apple, about 150 gms.; egg, about 65 gms. This was followed in every case except in that of the feeding of the concentrate-tartrate mixture in which the diet included a constant added quantity of apple, lettuce and milk. Such a basal ration was chosen so that the urine would be considerably more acid than normal. This made it possible to secure a wide range of variation of the urinary pH without producing an alkaline urine and the possible consequent varying equilibria.

It seemed desirable to adopt a given length of time for an experiment and to report all daily analyses. It would thus be possible to study the daily as well as the final influence of the food upon the composition of the urine. A total time of ten or eleven days was therefore decided upon. Each experiment was divided into two periods. For the first five days each person received only the basal ration. During the next five (or six) days each person received, in addition, a constant quantity of fresh grapes or some grape product.

Whereas previous investigators have used two subjects only, in these experiments with fresh grapes, whole grape juice and detartrated grape juice, three subjects were used. Two subjects were given the grape juice concentrate and one was kept on a shortened test of concentrate-tartrate mixture. Two subjects were used with each kind of raisin, giving a total of



four persons on these two very similar products. It appeared that, with such a plan of study, the results in general would be more reliable than with a smaller number of subjects.

Since Blatherwick (4) had used a 300 gm. daily portion of raisins, it seemed desirable to employ this quantity in the present study so that the results in both should be comparable. Consequently, approximately equivalent quantities, on a solids basis, of fresh grapes, grape juice, and grape juice concentrate were used. The daily portions were as follows: 1000 gm. fresh grapes, 1000 cc. natural grape juice, 1000 cc. detartrated grape juice, 300 gm. natural grape juice concentrate, and 40 gm. of the concentrate-tartrate mixture. Altogether, twelve young men connected with the institution and carrying on approximately the same daily schedules of work, recreation and sleep volunteered their services.

All analytical determinations were made in duplicate or triplicate, the average of closely agreeing duplicates only, being reported. The following methods were employed: pH, according to the method of Palmer, Salvesen, and Jackson (11) and by the quinhydrone electrode (average of colorimetric and electrometric determinations reported, since agreement of  $\text{pH} = 0.05$  was obtained); organic acids, Van Slyke and Palmer (17); ammonia, Van Slyke and Cullen (16); and titratable acidity, Henderson (9). Urine samples for 24-hour periods were collected, the experimental day ending just before breakfast each morning. The total volume was determined and 400 cc. specimens preserved with a few cc. of toluene. The pH determinations were made immediately, total acidity and organic acid determinations within twenty-four hours, and ammonia determinations within two or three days.

In addition to the urine analyses, determinations of the ash, the alkalinity of the soluble ash, and the alkalinity of the insoluble ash were made (1) on composite samples of the fresh grapes and on each of the grape products.

## RESULTS AND DISCUSSION

As is shown in Tables I to VI, the effect of the grapes and grape products on the pH of the urine is quite marked. On the basal diet the pH generally became stabilized at 5.85 to 5.70. It is interesting to note the different individual reactions shown in these experiments. For example, Subject C in Table I and Subject F in Table II remained at a pH of 6.0 on the basal ration; whereas several subjects showed a pH as low as 5.65. Since the data are continuous, it is possible to observe the nature of the daily changes occurring. It is apparent that this change is generally uniform with a tendency for pronounced increase of pH immediately following the initial in-

TABLE I  
EFFECT OF FRESH GRAPES ON COMPOSITION OF URINE

Day*	Subject A					Subject B					Subject C				
	Volume	pH	0.1-N Organic acids	0.1-N Titratable acids	Am- monia N	Volume	pH	0.1-N Organic acids	0.1-N Titratable acidity	Am- monia N	Volume	pH	0.1-N Organic acids	0.1-N Titratable acidity	Am- monia N
	cc.		cc.	cc.	mg.	cc.		cc.	cc.	mg.	cc.		cc.	cc.	mg.
1	1100	5.85	590	116	55	735	5.65	474	160	54	990	6.50	337	127	89
2	1130	5.90	445	79	70	1110	5.80	468	175	81	1265	6.00	623	89	99
3	1215	6.15	572	78	88	950	6.00	401	84	59	610	6.00	459	55	99
4	1280	5.60	633	225	108	1390	5.60	643	234	101	840	6.20	547	145	216
5	1250	5.70	603	165	105	1870	5.65	755	176	115	670	6.00	385	99	180
6	1690	5.60	558	220	85	1460	5.60	546	196	57	1125	6.45	651	153	69
7	1520	6.30	645	37	51	1440	5.95	615	130	64	1430	6.75	560	75	66
8	1540	6.65	620	34	43	1300	6.25	702	125	65	780	6.55	615	49	48
9	1785	6.60	757	14	40	1900	6.60	836	45	63	980	6.65	558	33	44
10	1680	6.75	703	17	38	900	6.60	415	49	35	1760	6.80	587	60	39

\* Days 1 to 5 inclusive, basal diet.  
Days 6 to 10 inclusive, basal plus 1000 gms. fresh Malaga grapes.

TABLE II  
EFFECT OF WHOLE GRAPE JUICE ON COMPOSITION OF URINE

Day*	Subject D					Subject E					Subject F				
	Volume	pH	0.1-N Organic acids	0.1-N Titratable acidity	Am- monia N	Volume	pH	0.1-N Organic acids	0.1-N Titratable acidity	Am- monia N	Volume	pH	0.1-N Organic acids	0.1-N Titratable acidity	Am- monia N
	cc.		cc.	cc.	mg.	cc.		cc.	cc.	mg.	cc.		cc.	cc.	mg.
1	890	6.05	475	93	90	2510	6.55	864	151	211	2060	5.90	667	140	173
2	1000	5.65	480	144	78	3605	5.90	577	144	202	2770	6.15	586	66	140
3	1200	5.80	484	150	88	2000	5.70	640	192	101	2960	6.25	698	71	133
4	820	5.80	436	100	78	1140	5.90	506	146	115	2100	5.90	680	164	129
5	940	5.85	489	98	99	1900	5.65	695	186	160	2460	6.00	674	118	138
6	970	5.90	654	169	109	1130	6.15	604	185	133	1930	6.60	710	97	97
7	1710	6.05	622	144	96	1010	6.05	540	208	91	1620	6.10	697	172	91
8	1750	6.50	514	99	59	4030	6.60	975	65	90	1860	6.10	614	138	83
9	1810	6.60	760	44	61	1650	6.60	828	33	56	1560	6.60	715	17	78
10	1720	6.65	760	21	67	2000	6.50	804	32	45	2150	6.50	855	47	72
11	1050	6.45	572	20	70	—	—	—	—	—	2660	6.65	783	53	74

\* Days 1 to 5 inclusive, basal diet.  
Days 6 to 10 inclusive, basal diet plus 1000 cc. whole grape juice.

TABLE III  
EFFECT OF DETARTRATED GRAPE JUICE ON COMPOSITION OF URINE

Day*	Subject D					Subject G					Subject H				
	Volume	pH	0.1-N Organic acids	0.1-N Titratable acidity	Am- monia N	Volume	pH	0.1-N Organic acids	0.1-N Titratable acidity	Am- monia N	Volume	pH	0.1-N Organic acids	0.1-N Titratable acidity	Am- monia N
	cc.		cc.	cc.	mg.	cc.		cc.	cc.	mg.	cc.		cc.	cc.	mg.
1	900	6.45	526	78	81	830	6.40	474	32	65	1140	6.20	595	50	53
2	1025	6.05	574	33	69	790	5.80	588	128	117	1140	6.05	514	110	70
3	890	5.85	549	126	80	900	5.85	577	139	111	1610	6.00	622	106	81
4	860	5.85	484	122	96	960	5.80	533	167	140	1770	5.80	610	181	99
5	950	5.85	527	139	91	1170	5.85	592	157	131	1550	5.85	459	135	69
6	1860	6.05	596	112	94	1185	6.00	685	211	179	1520	5.95	572	110	85
7	1800	6.40	572	86	101	1630	6.40	640	95	91	1690	6.20	514	101	66
8	1620	6.45	649	84	63	2015	6.40	660	52	102	1830	6.25	610	77	102
9	1100	6.20	495	90	71	1680	6.40	655	81	118	2100	6.40	651	50	71
10	720	6.00	636	76	68	1180	6.00	560	135	96	1940	6.40	637	43	74
11	710	6.25	530	14	20	1730	6.40	643	138	85	—	—	—	—	—

\* Days 1 to 5 inclusive, basal diet.

Days 6 to 10 inclusive, basal diet plus 1000 cc. detartated juice.

TABLE IV  
EFFECT OF CONCENTRATE AND CONCENTRATE-TARTRATE MIXTURE ON COMPOSITION OF URINE

Day*	Subject I (Concentrate)					Subject D (Concentrate)					Subject F (Concentrate-Tartrate)				
	Volume	pH	0.1-N Organic acids	0.1-N Titratable acidity	Ammonia N	Volume	pH	0.1-N Organic acids	0.1-N Titratable acidity	Ammonia N	Volume	pH	0.1-N Organic acids	0.1-N Titratable acidity	Ammonia N
	cc.		cc.	cc.	mg.	cc.		cc.	cc.	mg.	cc.		cc.	cc.	mg.
1	815	6.10	531	82	123	775	6.15	413	54	83	—	—	—	—	—
2	1250	5.85	520	95	118	820	5.85	611	126	98	2130	6.45	647	102	119
3	1380	5.90	607	61	77	1390	6.10	600	111	78	1940	6.60	655	39	87
4	1170	5.80	503	138	121	890	6.10	526	99	80	1020	6.40	600	50	49
5	1730	5.80	575	62	145	690	5.60	500	129	70	1500	6.40	639	61	51
6	1640	6.25	846	49	119	1270	6.05	465	127	85	2340	6.90	842	56	53
7	1760	6.45	598	85	109	960	6.30	588	48	59	1700	7.15	1280	88	28
8	1620	6.40	954	62	100	1100	6.55	714	35	49	1750	7.20	1021	91	20
9	1410	6.65	825	61	71	1760	6.80	587	60	39	—	—	—	—	—
10	1490	6.60	677	60	83	1050	6.80	624	42	47	—	—	—	—	—
11	2290	6.65	784	51	64	—	—	—	—	—	—	—	—	—	—

\* Days 1 to 5 inclusive, basal diet.  
Days 6 to 10 inclusive, basal diet plus 300 gm. concentrate.  
Days 6 to 8 inclusive, basal diet plus 40 gm. concentrate-tartrate mixture.

gestion of the grapes or grape product. In all cases there is a distinct total increase in the value of the pH, whether the pH of the initial period was, for example, 5.9 or 5.6.

In general, this increase was such that the pH on the last day with the basal ration plus grape product was 0.8 to 1.0 pH unit higher than that of the last day with only the basal ration. In these experiments, this was true for fresh grapes, natural fresh grape juice, untreated fresh grape juice con-

TABLE V  
EFFECT OF THOMPSON SEEDLESS RAISINS ON COMPOSITION OF URINE

Day*	Volume	pH	0.1-N Organic acids	0.1-N Titra- table acidity	Am- monia N	Volume	pH	0.1-N Organic acids	0.1-N Titra- table acidity	Am- monia N
	Subject J					Subject B				
	cc.		cc.	cc.	mg.	cc.		cc.	cc.	mg.
1	910	6.60	499	117	104	1050	5.95	391	177	73
2	980	5.85	499	110	93	1380	5.65	762	100	116
3	1125	5.70	550	158	76	1050	5.65	517	155	71
4	1230	5.70	542	187	89	1020	5.55	505	149	57
5	1140	5.65	570	194	115	1410	5.70	561	105	79
6	1070	5.75	665	178	126	1820	5.85	695	160	82
7	840	5.85	753	192	99	1270	6.45	707	95	93
8	1000	6.15	602	142	95	1020	6.20	564	78	80
9	1120	6.45	765	40	56	1170	6.40	794	65	53
10	1240	6.40	905	47	58	1220	6.50	856	62	48
11	1190	6.40	800	67	38	1430	6.30	616	60	56

\* Days 1 to 5 inclusive, basal diet.

Days 6 to 11 inclusive, basal diet plus 300 gm. raisins.

centrate, and for Thompson seedless and Muscat seeded raisins. As indicated in Table VI, even 100 grams of Muscat seeded raisins are effective in decreasing the reaction of the urine.

With the detartrated grape juice, the average increase of pH was only 0.5 to 0.6 units, or about one-half that produced by the untreated fresh juice, concentrate and raisins. With the concentrate-tartrate mixture the pH increase was approximately as great as with the natural juice, concentrate and raisins but the large quantity of added tartrate in the mixture rendered it unpalatable and the study was not continued after the third day. Even in this experiment, the excess of base in the grape product is

sufficient to balance the organic acidity and to reduce greatly the acid reaction of the urine.

From the data in Tables I to VI showing the values for total acids and ammonia excreted daily, it is apparent that there was a marked decrease in these factors when the grapes or grape products were added to the basal diet. In the experiment with the concentrate-tartrate mixture the effect was not so decided although with even the excessive amounts of tartrate

TABLE VI  
EFFECT OF MUSCAT SEEDED RAISINS ON COMPOSITION OF URINE

Day*	Volume	pH	0.1-N Organic acids	0.1-N Titra- table acidity	Am- monia N	Volume	pH	0.1-N Organic acids	0.1-N Titra- table acidity	Am- monia N
	Subject A					Subject H				
	cc.		cc.	cc.	mg.	cc.		cc.	cc.	mg.
1	870	6.70	592	174	59	1420	6.30	579	80	96
2	860	5.80	509	114	77	1920	5.70	661	146	129
3	1480	5.80	474	161	86	920	5.65	486	184	93
4	1820	5.80	641	197	102	1240	5.65	536	213	97
5	1020	5.75	443	150	96	1180	5.75	560	196	124
6	1520	6.15	995	128	102	1110	5.80	688	158	100
7	820	6.05	563	120	64	1470	6.25	443	62	91
8	830	6.00	990	113	56	1040	6.25	604	95	47
9	950	6.30	820	72	43	1150	6.50	816	55	39
10	950	6.85	650	27	32	1240	6.50	832	47	55
11	980	6.70	611	8	27	1130	6.55	650	45	44

\* Days 1 to 5 inclusive, basal diet.

Days 6 to 9 inclusive, basal diet plus 300 gms. raisins.

Days 10 to 11 inclusive, basal diet plus 400 gms. raisins.

ingested, the total acid increase is only equal to the drop in the ammonia excreted.

A further significance of these results is obtained from a consideration of the relationship between the alkaline reserve and the acid and ammonia excretions. Fitz and Van Slyke (7) have shown that in normal men "the excretion of acid in excess of fixed bases as measured by determining the ammonia and titratable acid bears a quantitative relationship to the alkaline reserve of the body as measured by the CO<sub>2</sub> binding power of the blood plasma." They have developed an evaluation of this relationship which is

$$\sqrt{D/W\sqrt{C}}$$

where D represents the rate of excretion of 0.1 N ammonia plus titratable acid per 24-hour time unit, C the 0.1 N  $\text{NH}_3$  plus acid per liter of urine and W the body weight. They assume the acid accumulation in the plasma is proportional to the fall of the plasma  $\text{CO}_2$  figure below the maximum plasma  $\text{CO}_2$  capacity expressed in volumes per cent. This fall of the plasma  $\text{CO}_2$  is then given by the expression above. Consequently, as  $\sqrt{D/W\sqrt{C}}$  increases, there is a corresponding decrease in the plasma  $\text{CO}_2$  and an indication of the change of the plasma  $\text{CO}_2$  level is obtained. This affords an estimate of the decrease of the alkaline reserve. In their studies with normal individuals, the value of the alkaline reserve as determined in the urine usually had an average error of 3.2 volume per cent.

Using the values for titratable acid, ammonia, and total volume given in Tables I to VI and the curves of Fitz and Van Slyke (7), the values for the index  $\sqrt{D/W\sqrt{C}}$  were computed. These are given in Table VII. Within the limits of accuracy, it is apparent that the increase of the index was general in all the basal diet periods. Correspondingly, there was a marked decrease in the index when the grapes or grape products were added to the basal diet. In most cases the grapes or grape products decreased the index by one-half or more. This decrease is an average index of 2.8 volumes per cent below the maximum plasma  $\text{CO}_2$  capacity. If this maximum is taken as 80 volumes per cent (7) and the normal value for all experiments as 80 volumes per cent minus the average index for the first day, or 74.7 volumes per cent, it is evident that with a final average value of 77.2 volumes per cent the plasma  $\text{CO}_2$  capacity has been increased above the normal by the grapes and grape products. The exception was that of Subject G with the detartrated juice in which case the total decrease of the index was nevertheless very marked. It may therefore be concluded that grapes and grape products such as were employed in these experiments, assist in securing a high alkaline reserve as estimated by the plasma  $\text{CO}_2$  binding power determined from the acid and ammonia content of the urine.

A contrast to the results obtained in these experiments is presented by those of Pickens and Hetler (12) who have reported that no marked change occurred in the acidity of the urine. Their published data are very condensed, neither the results of the daily analyses nor the values of the urinary ammonia and total volumes being given. It is, therefore, impossible to observe either the general daily trend or to calculate any other data from their results.

It was known that the California grapes and grape products which are here shown to be markedly basic in physiological reaction also yielded an



TABLE VII  
INDEX OF LOWERING OF PLASMA CO<sub>2</sub> CAPACITY  
(Volumes Per Cent)

Day	Fresh grapes	Fresh grapes	Fresh grapes	Whole grape juice	Whole grape juice	Whole grape juice	Detartarated grape juice	Detartarated grape juice	Detartarated grape juice	Grape juice concentrate	Concentrate-tartrate	Thompson seedless raisins	Thompson seedless raisins	Muscata seedless raisins	Muscata seedless raisins	
	A	B	C	D	E	F	D	G	H	I	D	F	J	B	A	H
1	5.0	6.3	5.4	5.0	6.1	6.8	4.9	3.1	3.0	6.4	4.5	—	6.6	6.5	6.9	4.3
2	4.8	6.3	4.9	6.1	6.0	4.2	3.5	6.8	4.9	5.7	6.5	5.1	5.6	5.0	5.9	5.6
3	4.9	4.1	4.9	6.0	5.7	4.0	6.0	6.5	4.4	4.4	5.2	3.3	5.9	6.0	6.1	6.7
4	8.0	7.3	8.1	5.8	6.0	6.0	6.1	7.2	6.0	7.5	5.3	3.4	6.4	5.8	6.8	7.3
5	7.0	6.2	5.0	5.4	6.6	5.1	6.4	7.1	5.0	5.2	7.0	3.3	7.4	5.0	6.6	7.1
6	7.0	6.0	5.5	8.4	7.0	4.5	5.0	8.1	4.9	5.1	5.8	2.7	7.4	5.5	5.8	6.1
7	3.0	5.0	3.9	5.5	7.0	6.5	4.7	4.6	4.0	5.0	4.3	3.1	8.0	4.9	5.6	3.8
8	2.0	4.9	3.1	4.9	2.5	5.5	4.3	4.0	4.5	4.7	3.0	3.1	6.6	4.5	5.1	3.9
9	1.6	2.9	3.0	3.0	2.1	3.0	4.5	4.7	3.0	4.2	3.0	—	3.0	3.2	4.0	3.1
10	1.5	2.8	3.0	2.5	2.0	3.0	4.8	6.0	2.9	4.3	2.9	—	3.1	3.1	2.1	3.1
11	—	—	—	2.5	—	3.7	1.8	5.5	—	3.6	—	—	3.1	3.1	1.1	2.8

ash that was highly alkaline. It therefore seemed desirable to learn whether there was any correlation between the alkalinity of the ash of the juice or grape product and the body reaction. Hartman and Tolman (8) have published the results of a comprehensive study of the chemical effects of the manufacture of Concord grape juice. Their studies were made in the districts in which the great bulk of such commercial juice is manufactured. Consequently their results are authentic and satisfactory for the purpose of comparison. Their averages of a great many determinations are taken to approximate the values for the commercial Concord juice employed by Pickens and Hetler. Their average results are given in Table VIII, to-

TABLE VIII  
CHEMICAL COMPOSITION OF GRAPES AND GRAPE PRODUCTS

Materials	Ash per 100 gm.	Alkalinity soluble ash, N/10 Acid, per 100 gm.	Alkalinity insoluble ash, N/10 Acid, per 100 gm.	Total alkalinity N/10 Acid, per 100 gm.
	gms.	cc.	cc.	
Concord grape juice—at time of storing . . .	0.39	41.1*	7.5*	48.6*
Concord grape juice—after 4 month's storage	0.27	28.3*	4.0*	32.3*
Concord grape juice—after 16 month's storage	0.24	21.8*	5.9*	27.7*
Malaga grapes—fresh . . . . .	0.60	77.1	31.2	108.3
Grape juice—fresh, used in diet . . . . .	0.40	39.0	35.2	74.2
Grape juice—detartrated, used in diet . . . .	0.27	20.8	23.0	43.8
Grape juice concentrate, used in diet . . . .	1.33	152.0	94.5	246.5
Concentrate-tartrate mixture, used in diet	18.4	2430.0	770.0	3200.0
Thompson raisins, used in diet . . . . .	2.20	218.0	121.0	339.0
Muscat raisins, used in diet . . . . .	2.07	234.0	106.0	340.0

\* cc. N/10 Acid, per 100 cc. juice.

gether with those of grapes and the various grape products used in the present study. It will be noted that the values for the alkalinities of the Concord juices are for 100 cc. quantities while all others are for 100 grams. The relative differences are probably small in this case. The total alkalinity of the Concord grape juice decreases about 33 per cent during the first four months' storage and about 43 per cent during the entire first sixteen months' storage, resulting in a final average alkalinity of 27.7 cc. 0.1 N acid per 100 cc.

In sharp contrast to this alkalinity is that of the juice used in the present experiment of which the alkalinity is approximately 74 cc. 0.1 N acid per 100 gram. The detartrating of this fresh juice resulted in a decrease of the

total alkalinity of about 41 per cent of the original to a value of about 44 cc. 0.1 N acid per 100 grams. The decrease in total alkalinity of both juices is approximately the same for the two methods. However, the final total alkalinity of the detartrated juice used in the present diets is still nearly as high as that of the fresh Concord juice at the time of storing and before it was detartrated. The juices being compared then have the following approximate alkalinities as cc. 0.1 N acid per 100 grams: fresh grape juice used in the present experiment, 74; detartrated grape juice similarly used, 44; and 28 for the Concord grape juice as prepared for commercial use.

If the magnitude of the basic reaction of the body parallels that of the quantity of basic food ingested only in addition to a certain fixed basal ration, this basic reaction of the body is a consequence of the basic food so ingested. It has been shown here that with every experiment with fresh grape juice, as well as with grapes and certain grape products, the reaction of the body has become markedly basic in comparison to the normal reaction estimated from the analyses of the first day and before new equilibria had been effected. It is also evident that the detartrated grape juice used in these experiments and having somewhat more than one-half as basic an ash as the fresh juice, did not produce such a marked basic reaction, but that there was, nevertheless, a distinct basic effect. In fact, the average basic effect of the detartrated grape juice used here appeared to be approximately one-half that of the fresh grape juice and grape products of equivalent basic ash content. Further, it has been shown (12) that a Concord product with somewhat more than one-third the basic ash content (8) did not produce a marked basic reaction of the body. Since such a parallelism does exist, there is evidently a definite relationship between the body reaction and the alkalinity of the ash of grapes and grape products. The results here reported demonstrate that this is true for grapes, fresh grape juice, detartrated grape juice, fresh grape juice concentrate, Thompson seedless and Muscat seeded raisins of the types investigated.

From such a study it is evident that the divergency of the results of Blatherwick with raisins and those of Pickens and Hetler with a commercial Concord grape juice should be expected. This is because of the very marked difference in their respective basic properties as indicated by the alkalinity of the ash.

A study of the quantitative relationship between the alkalinity of the ash and the body reaction should be fruitful. Such a study will be reported in another paper from this laboratory.

From Tables I to VI inclusive, it is evident that there is an increased excretion of organic acids coincident with the decrease of both the total

TABLE IX  
ORGANIC ACIDS INGESTED AND OXIDIZED

Subject	Fresh grapes			Whole grape juice				Detartarated grape juice			Grape juice concentrate		Concentrate	Thompson seedless raisins		Muscat seeded raisins			
	A	B	C	D	E	F		D	G	H	I	D	F	J	B	A		H	
Av. daily organic acid titration for basal diet period (cc. N/10 HCl)	565	548	470	473	656	661		532	553	560	547	530	635	532	547	532	400 gm.	300 gm.	400 gm.
Av. daily organic acid titration for period with basal diet+grapes, etc.	657	623	594	647	750	729		580	639	597	781	596	1047	748	705	771	630	672	741
Difference (cc. N/10 HCl)	93	75	124	164	94	68		48	86	37	234	66	412	216	158	239	98	108	177
Organic acids not oxidized (gm.)	1.02	0.85	1.34	1.68	1.05	0.76		0.53	0.96	0.41	2.50	0.71	4.58	2.41	1.76	2.66	1.09	1.20	1.97
Organic acids ingested daily (gm.)	27.8	27.8	27.8	22.2	22.2	22.2		12.7	12.7	12.7	31.4	31.4	36.1	24.1	24.1	23.7	31.7	23.7	31.7
Per cent oxidized	96.3	97.0	95.0	92.4	95.3	96.6		95.8	92.4	96.7	92.0	97.7	87.3	90.0	92.7	88.8	96.6	94.9	93.8

acidity and ammonia. This is similar to the effect of orange juice (5). This was explained as being probably the result of a part of the citric acid escaping oxidation in the body and thus being present in the urine and increasing the titration value for organic acids. The same explanation may apply in the case of the organic acids in grapes and grape products.

The organic acid content of the grapes and grape products was determined by the method of Van Slyke and Palmer (17). The total amounts daily ingested in each experiment are given in Table IX. The differences of the average daily organic acid titration of the urine with and without the grapes or grape products added to the basal diet are also given. From these values, the percentage of the organic acids oxidized in the body may be computed.

For example, the data with the fresh grapes may be considered. The Van Slyke and Palmer titration indicated that the organic acid content was equivalent to a concentration of 1.88 per cent as tartaric acid. Since by this method tartaric acid is titrated to the extent of about 67 per cent, the true value for total tartaric acid would be about 2.8 per cent. Therefore, approximately 28 grams would be taken daily by an individual. The average daily increase of excreted organic acids in the urine of the last five days above that of the first five, for Subject A with fresh grapes, was equivalent to 92 cc. of 0.1 N acid. Assuming that this increase was entirely due to tartaric acid, such acid in the urine would be about 1.02 grams. Therefore, approximately 3.7 per cent of the ingested tartaric acid of the fresh grapes appears to have escaped oxidation.

In this manner the total amount of organic acids ingested, the amount excreted, and the per cent organic acids oxidized, have been calculated and presented in Table IX. In each experiment with fresh grapes, whole grape juice, detartarated grape juice, whole grape juice concentrate, Thompson seedless and Muscat seeded raisins, the individual ability to oxidize the ingested organic acids is high, varying from approximately 90 to 97 per cent. An average of the sixteen experiments with varying quantities of total ingested tartrate indicates that approximately 94.4 per cent of the organic acid, as tartaric, is oxidized. For the concentrate(—)tartrate mixture with a very excessive amount of tartrate present, over 87 per cent of the tartrate, as tartaric acid, is oxidized. The average with all experiments of approximately 94 per cent oxidation compares quite interestingly with that of Blatherwick and Long (5) who reported approximately 94 per cent oxidation of citrates or organic acids, as citric acid, in experiments with orange juice.

## SUMMARY

Sixteen experiments with men subjects on basal diet and on the same basal supplemented by fresh grapes, fresh natural grape juice, detartrated grape juice, natural grape juice concentrate, concentrate-tartrate mixture, Thompson seedless and Muscat seeded raisins are reported. The following results were observed when the grapes or grape products were added to the basal ration.

1. An increase in the pH of the urine occurred, ranging from 0.8 to 1.2 pH units.

2. A decrease in the ammonia excreted and a corresponding decrease in the total acidity was noted.

3. There was an increase of the alkaline reserve above the normal for each subject, the alkaline reserve being estimated by the plasma  $\text{CO}_2$  combining power determined from the acid and ammonia content of the urine and calculated by the method of Fitz and Van Slyke.

4. A correlation between the alkalinity of the ash and the physiological reaction was apparent. Grapes and grape products with the greater alkalinity of ash were associated with a more basic body reaction.

5. An increase occurred in organic acids excreted. This may be explained by the presence in the urine of some incompletely oxidized tartaric acid.

6. Approximately 94 per cent of the ingested organic acids appeared to be oxidized.

These results are applicable to grapes and grape products of the varieties and types investigated.

The writer wishes to express his appreciation of the interest and thoughtful consideration of Professor W. V. Cruess, upon whose suggestion this study was initiated. The friendly advice of P. F. Nichols of this laboratory has been of great assistance. The full cooperation of the men taking a part in the diets is acknowledged with pleasure.

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## A STUDY OF THE CALCIUM RETENTION ON A DIET CONTAINING AMERICAN CHEDDAR CHEESE

By

MARGUERITE G. MALLON, L. MARGARET JOHNSON, AND CLARA R. DARBY  
(*From the Department of Foods and Nutrition, School of Home Economics,  
Purdue University, Lafayette, Indiana*)

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COW'S milk has been generally recognized as a good source of calcium in the diet. Unfortunately, individuals often dislike milk, and since the commonly used American Cheddar cheese is made from cow's milk, it would seem of value to determine whether its calcium is as well utilized as the calcium of milk. From a survey of literature the experiments reported on the digestibility of cheese (1, 2, 3, 4) seem to indicate that cheese is well utilized. However, there do not appear to be any experiments reported on the calcium retention of American Cheddar cheese or of any other variety of cheese.

Cheese made by different methods, that is, with rennet and with lactic acid, contains different percentages of calcium as reported by Blunt and Sumner (5). They found the average per cent of calcium in American Cheddar cheese to be 0.71 per cent; Swiss cheese 1.05 per cent; while that in cottage cheese was 0.077 per cent. The calcium content of the American Cheddar cheese used in this calcium retention study was found to be of similar percentage namely, 0.74 per cent.

A compilation of the data of four investigators (6, 7, 8, 9) on the retention by adults of calcium of pasteurized whole milk is given in Table I.

Two healthy young women, one an assistant in Nutrition in the School of Home Economics and the other a graduate student in Nutrition, served as the subjects. They were comparatively similar in weight, namely, 51.3 kilograms and 50.2 kilograms. They were well within the correct range of weight for their height, had lived on an adequate diet previous to this study, and lived healthy, active lives. During this study the subjects were engaged in the same activities and were not exposed to direct sunlight.

The studies extended over a period of 18 consecutive days during February. The 18 days were divided into two periods of 9 days each, one for the study of American Cheddar cheese and the other for the study on pasteurized milk. Each 9-day period consisted of a 3-day preliminary period followed by a 6-day experimental period. During the preliminary period the same weighed amounts and kinds of food were eaten as those of the experimental period.



The diets were planned to furnish an amount of calcium that would be close to the calculated average requirement for equilibrium (10) and to meet the protein and energy requirements of the subjects. The diet for the study of the calcium retention of cheese consisted of American Cheddar cheese, lean round beef, cored apple, soda crackers, butter, and sucrose. In the milk study the diet was the same except that pasteurized milk replaced the American Cheddar cheese. Distilled water was used *ad libitum* for drinking.

TABLE I  
COMPILATION OF CALCIUM STUDIES ON PASTEURIZED MILK

Investigator	Subjects adult women	Body weight	Calcium from milk	Intake per kg. per day	Calcium balance per day	Calcium balance per kg. per day
		kg.	per cent	mg.	gm.	mg.
Rose* (1920)	E. B.	54	70	7.1	+ .060	+1.1
	R. E.	56	70	6.8	+ .087	+1.5
Rose and MacLeod* (1923)	B. B.	54	70	6.0	- .024	-0.4
	G. C.	62.5	74	6.4	+ .008	+0.1
	C. M.	45	71	6.6	- .039	-0.8
Willard and Blunt (1927)	3 adults—1 serving twice; making 4 comparisons	no weights given	50	no data	- .09 - .16 - .01 - .03	no data
Kramer, Latzke, Shaw (1928)	E. L.	53.6	68	9.4	- .242	-4.5
	I. B.	58.2		8.6	- .122	-2.1
	A. L.	68		7.4	- .125	-1.8
	M. K.	56.3		8.9	- .098	-1.7

\* Personal communication.

The American Cheddar cheese<sup>1</sup> was made in September, 1930. "The milk from which the cheese had been made was pasteurized at 160°F and was not held at this temperature for any length of time." The vitamin A content of the same American Cheddar cheese as used in this study was tested in the Nutrition Laboratory and it was shown that 0.5 gram per day was as effective as 0.8 and 1.0 gram in the prevention of eye disease and in the production of normal growth in albino rats. Sherman's (11) quantitative method was used.

<sup>1</sup> Obtained through the courtesy of Professor H. W. Gregory, Department of Dairy Husbandry, Purdue University, from the Kraft Phenix Cheese Company at Marion, Indiana.

The milk, consumed as purchased, was obtained fresh each day from the Purdue University Creamery. It was milk that had been pasteurized at 142° to 145°F, held at that temperature for at least 30 minutes, then cooled immediately to 50°F or less.

The calcium from the cheese furnished 86.0 per cent of the total calcium of the diet for one subject and 85.7 per cent of the total calcium for the other. The calcium from the milk furnished 87.7 per cent of the total calcium of the diet for one subject and 87.4 per cent for the other.

A sufficient amount of food, with the exception of milk, butter, and meat, was purchased at one time for both experiments. The meat was lean round of beef purchased from the local market in two lots. It was trimmed of fat, ground, and well mixed. Samples were taken for calcium analysis. A day's supply for each subject was weighed for the entire experimental period, wrapped in oil paper, and kept in cold storage. The meat was baked in casseroles in a hot oven from 10 to 20 minutes, according to the preference of the subject. It was eaten from the same container in which it was baked in order to avoid loss. The apples, of the winesap variety, chosen for flavor, were purchased from the local market and were kept in cold storage. To meet the caloric requirement, sugar was eaten as pure cane sugar made into a flavored paste. The daily supply of sucrose was evenly divided for the three meals. The protein of the diet was of a high biological value and was consumed in the amount of at least one gram per kilogram of body weight. The Torsion balance used for the weighing of the food consumed had its weights checked to the second decimal place with quantitative weights.

The daily intake of calcium is given in Table II. In the cheese study, the average daily calcium intake for each subject was 10.2 milligrams per kilogram of body weight; and in the milk study, 9.4 milligrams per kilogram. These amounts were sufficient for calcium equilibrium for women subjects as indicated by the work of other investigators (9, 12, 13, 14, 15).

For the calcium determinations weighed samples of American Cheddar cheese, pasteurized milk (a composite sample for every three days), cored apple, lean ground beef, and soda crackers were dried in an electric oven and then ashed in porcelain crucibles according to the official method (16) using an electric muffle furnace, kept between 400°C and 450°C.

Urine and feces of the 3-day preliminary period were not collected. The first collection of the excreta was made the first day of the experimental period. The feces were dried and ashed for calcium analysis.

Calcium of food, feces, and urine was determined by McCrudden's method (17) with the *pH* value adjusted according to Shohl and Pedley

(18). The 0.01N and 0.05 N  $\text{KMnO}_4$  solutions were prepared and standardized according to Halverson and Bergeim (19). The potassium permanganate solution used in any series of calcium determinations was restandardized before the titrations for calcium were made. For titrations U. S. Bureau of Standard burettes were used. Redistilled water was used for the preparation of all solutions and for rinsing of all apparatus used in making

TABLE II  
DAILY FOOD INTAKE

Food	American Cheese Experimental Period				Pasteurized Milk Experimental Period			
	Subject I (51.3 kg.)		Subject II (50.2 kg.)		Subject I (51.3 kg.)		Subject II (50.2 kg.)	
	Wt.	Calcium	Wt.	Calcium	Wt.	Calcium	Wt.	Calcium
Cheese, Amer. Cheddar	gm. 61	gm. .450	gm. 59.5	gm. .439	gm. —	gm. —	gm. —	gm. —
Milk, whole pasteurized	—	—	—	—	338	.421	330	.411
Apples, fresh, cored but unpared	300	.014	300	.014	300	.014	300	.014
Beef, lean round	150	.022	150	.022	150	.008	150	.008
Crackers, soda	75	.031	75	.031	75	.031	75	.031
Butter	40	.006*	40	.006*	40	.006*	40	.006*
Sucrose	200	—	180	—	200	—	180	—
Total		.523		.512		.480		.470

\* Rose, M. S., Laboratory Handbook for Dietetics, 3rd edition 1929.

the analyses. The chemicals used were secured from the Mallinckrodt Chemical Works. The modified calcium method was subjected to preliminary testing for the recovery of calcium from known solutions. Determinations of calcium were made in triplicate.

In Table III are given the average daily calcium intake and output for the subjects for both the American Cheddar cheese study and the pasteurized milk study.

*Discussion and Results*

The calcium balances, as given in Table III, show a daily average balance of +0.005 grams for Subject I and a daily average balance of -0.040 grams for Subject II on the diet in which American Cheddar cheese was used; and the calcium balances show a daily average balance of -0.010 grams for Subject I and a daily average balance of -0.031 grams for Subject II on the diet in which pasteurized whole milk was used.

TABLE III  
CALCIUM BALANCE (6 DAYS)

Subject	Experimental period	Calcium Intake		Calcium Output			Balance	Balance per day	Balance per kg. per day
		total	per kg. body weight	urine	feces	total			
Subject I weight 51.3 kg.	Cheese, Amer. Cheddar	gm.	mg.	gm.	gm.	gm.	gm.	gm.	mg.
		3.138	10.2	1.099	2.012	3.111	+0.027	+0.005	+0.1
	Milk, whole pasteurized	2.871	9.4	0.590	2.343	2.933	-0.062	-0.010	-0.2
Subject II weight 50.2 kg.	Cheese, Amer. Cheddar	3.072	10.2	0.538	2.774	3.312	-0.240	-0.040	-0.8
	Milk, whole pasteurized	2.811	9.4	0.484	2.524	3.008	-0.187	-0.031	-0.6

Comparing the calcium balances of Subject I on the two diets, the results indicate that Subject I changed from an almost negligible positive balance to a slight negative balance; i.e., Subject I remained almost in calcium equilibrium. On both the cheese and pasteurized milk diets, Subject II showed slightly negative balances of approximately equal value.

It would seem, therefore, if Subject I were almost in calcium equilibrium and Subject II showed negative calcium balances of approximately the same value, that the calcium of the cheese was as well utilized as the calcium of the milk.

During one day of the cheese experimental period Subject II had four bowel movements instead of the usual one. The two young women subjects maintained their weight and remained in good health throughout the experiment.

The results of this experiment, in which two healthy young women served as subjects, seem to indicate that the calcium of the American Cheddar cheese was as well utilized as the calcium from the pasteurized whole milk.

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# A QUANTITATIVE STUDY OF THE DIETARY OF THE HUMAN MOTHER WITH RESPECT TO THE NUTRIENTS SECRETED INTO BREAST MILK\*

By

CARROLL F. SHUKERS, ICIE G. MACY, BETTY NIMS,  
EVA DONELSON, AND HELEN A. HUNSCHER†

*(From the Nutrition Research Laboratories of the Merrill-Palmer  
School and the Children's Hospital of Michigan,\*\* Detroit.)*

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THE present paper reports an investigation of the self-chosen diets and the nutritive requirements of three multiparae during 60 weeks of intensive lactation, with special reference to the intake of calories, proteins, fats, carbohydrates, calcium, and phosphorus in the food and the outgo of these substances in the milk secreted. These women produced an average of 1419 cc., 2366, and 3134 cc. of milk daily. In view of the current empiric regimens recommended for lactating women, it is pertinent to consider the quantity of nutrients actually utilized in the secretion of milk.

The principal organic constituents of milk, a secretion with a fairly constant chemical composition for each species, are casein and lactalbumin, milk fat, and lactose. These constituents, peculiar to milk, are secreted by the mammary glands from precursors such as amino acids, phosphatids, and glucose respectively, in the blood. All the organic and inorganic constituents of milk may be derived either from ingested food or from body tissue.

Though inorganic elements, vitamins, and some drugs and other substances appear to be transferred directly to milk, food proteins, fats, and sugars do not ordinarily follow this course (1 to 11).

Nevertheless, the woman who is producing an adequate amount of milk of good quality and doing so without any marked loss in body weight, must be transforming ingested food into milk. Meigs and Cary (12, p. 494) state:

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\*\* Now the Research Laboratories of the Children's Fund of Michigan, Detroit.

that

There is every reason to think, therefore, that, under ordinary circumstances, milk protein is made from food protein, milk fat from food fat, and milk carbohydrate from food carbohydrate. But it has also been clearly shown that this is not always the case, and the numerous other possible ways in which the mammary gland may get materials for the manufacture of its product must be kept clearly in mind in order to interpret the results of the numerous experiments which have been carried out to determine the effects of changes in the food supply on milk secretion.

It is known that some constituents are interconvertible in metabolism, and, this being so, milk fat and carbohydrate are not necessarily to be traced directly to the corresponding food substances. It has been demonstrated also that proteins may be transformed into glucose and glucose into fat (13, p. 267). Proteins, fats, and carbohydrates have been shown to serve as the initial source of milk fat in the lactating organism (14, 15). Since the mammary glands must compete with other organs for nutrients from the blood stream during the metabolic processes, there is a variation in the quantity and proportion of the various components available at any given time for milk synthesis. This variation is influenced by digestion, absorption, and environmental factors.

When the necessary ingredients for milk synthesis are lacking in the daily food, the mother may either decrease her milk output, secrete milk deficient in these constituents, or draw them from her own tissues. A marked deficiency of dietary proteins, fats, or carbohydrates is usually associated with a prompt drop in milk volume; inadequate vitamin intake may result in a vitamin-deficient milk; while an insufficient mineral intake results in a drain on the maternal reserves. Although intensive lactation is often marked by negative calcium and phosphorus balances (16, 17) there is obviously a limit to the amount of material a woman can lose from her tissues without some impairment of her body and a diminution in the nutritive value of her milk. The interrelations of these many dietary and metabolic factors in milk secretion and their application to the nutrition of the mother and child have been reviewed in detail elsewhere (12, 18, 19).

Experimental work to demonstrate the precursors of milk constituents and the factors determining the metabolic requirements for lactation, has been done chiefly on mammals other than man and especially on the cow and goat. Though the findings on these animals are of significance in indicating the general relationships, they cannot be expected to show the specific demands of woman. The digestive processes and the synthetic capacity of different species may vary (20). Investigations leading to a full knowledge of the processes of and requirements for lactation in woman will serve to replace our present empiric regimen for the lactating mother with a rational one. The present study may add apposite information in this direction.

### PROCEDURE

The subjects of this study were housewives carrying on their usual home duties. They had sustained continuous reproductive processes for a period of four to six years. Their diets were entirely self-chosen and consisted of those foods which they liked and which they believed to favor milk flow. The quantity of each food stuff ingested over intervals of 3 to 10 consecutive days was weighed and its composition calculated by recognized methods. The breast milk was expressed at 4-hour intervals throughout the experimental period and chemical analyses were made on the 24-hour composite samples. The detailed methods used in this study were described in preceding papers, where it was shown that these women chose diets that were liberal in quantity (21) and excellent with respect to quality and the proportions of the several nutrients (22). A comprehensive consideration of the volume output of breast milk (23) and its composition (24) has been reported elsewhere. The average daily milk output over a 60-week period during the fourth lactation for subject VI and the third for subjects VII and VIII was 3134, 2366, and 1419 cc., respectively. These women were apparently producing milk at their maximal capacity, as indicated by the ingestion of a liberal diet, the persistence in regular habits of good hygiene, and the regular, thorough, manual emptying of the breasts daily throughout the lactation period.

During this interval the body weight of subject VI decreased from 73.1 to 65.5 kilograms, that of subject VII decreased from 65.0 to 61.8 kilograms, and that of subject VIII increased from 70.4 to 81.8 kilograms.

### EXPERIMENTAL

*Calories.* During intense lactation the caloric exchange by a woman who is maintaining her weight is a striking example of the transformation of ingested food into milk nutrients. Hoobler (25) has shown that mothers receiving 2670 and 2774 calories per day produced milk containing 1096 and 1296 calories, respectively, or 41 and 47 per cent of the total food calories. The notable ability of some women to convert food energy into milk energy is further shown in Table I and Chart 1. Subjects VI, VII, and VIII ingested daily dietaries with caloric values of 4300, 4600, and 3800, respectively, and secreted milk containing 2100, 1500, and 1120 calories, respectively.<sup>1</sup> The caloric content of the breast milk in these cases was equivalent to 50, 33, and 30 per cent of the total intake. The percentage distribution of calories between proteins, fats, and carbohydrates in the

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<sup>1</sup> The mean food intakes cited in this paper represent the weighed food ingested on the days when breast milk was simultaneously collected.



diets of these women was in the ratio of 15:37:48, a proportion in agreement with that recommended by Rose (26, p. 25).

A comparison of the volume output with the caloric values of the three milks shows that the highest caloric value per liter, 770, was achieved by subject VIII, who produced the smallest volume of milk. However, the milk of subject VI had a caloric value of 680 per liter and that of subject VII only 660 per liter, although the former produced nearly 800 cc. more milk per day than the latter did. Hence, though the caloric value of the milk of these women showed individual variation, this variation was not necessarily related to volume.

TABLE I  
ESTIMATED AVERAGE DAILY CALORIC BALANCE

Subjects	Total 24-hour			Per sq. m. body surface			Per kg. body weight*		
	VI	VII	VIII	VI	VII	VIII	VI	VII	VIII
Food, Cal.....	4300	4600	3800	2500	2660	2090	65	73	49
Breast milk, Cal.....	2100	1500	1120	1200	870	590	30	25	14
Excreta loss, Cal.....	390	460	400	—	—	—	—	—	—
Surplus, Cal.....	1810	2640	2280	1070	1530	1270	27.3	42.0	29.4
Calories per sq. m. body surface per hour									
Milk value per liter, Cal...	680	660	770						
Average daily milk volume, cc.....	3134	2366	1419	Food.....			104	110	87
Outgo in milk as percentage of intake.....	50	33	30	Surplus.....			44.6	63.8	52.9

\* Change in body weight during lactation in kilograms: VI, 73.1 to 65.5; VII, 65.0 to 61.8; VIII, 70.4 to 81.8.

Inasmuch as milk volume is the chief determinant of the total output of milk calories, the milk volume becomes a factor of moment in planning diets for lactating women. The active mother who is putting 2000 calories daily into milk must obviously receive a much more abundant diet than is needed by the mother (27, p. 602) who is secreting only 700 calories or less into breast milk. A continued inadequate caloric intake leads to a diminished milk flow. On the other hand, caloric overfeeding does not stimulate milk production (18, 28).

Breast milk has a variable caloric value, depending largely on its fat content and to a lesser degree on its concentration of carbohydrate and protein. Schlossman (13, p. 548) examined nineteen samples of milk from as many women and found an average caloric value per liter of 719 (maxi-

imum of 876 and a minimum of 567). Morse (29, p. 121) states that the caloric value of breast milk is usually cited as 782 per liter, although the individual milks vary greatly. The calories of breast milk are derived from the cleavage products of fats, carbohydrates, and proteins from either ingested food or maternal tissue. There appears to be a definite tendency,

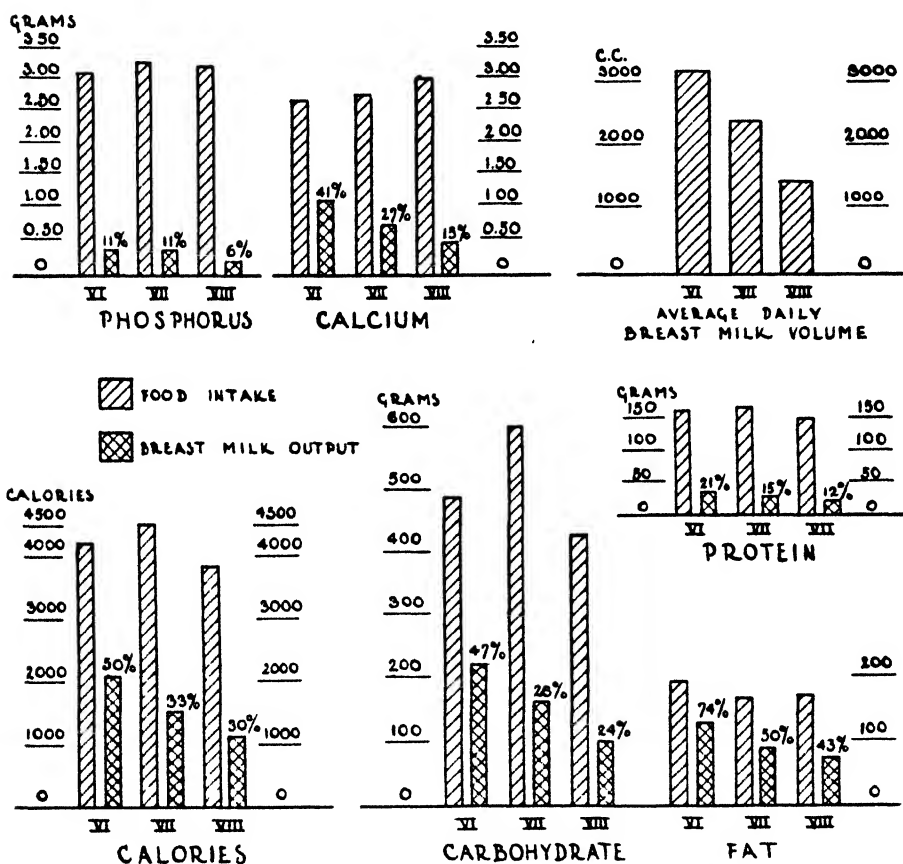


Chart 1.—Illustrates the average daily food intake and the outgo in breast milk of calories proteins, fat, carbohydrate, calcium and phosphorus.

especially in early lactation, to maintain milk secretion at the expense of the maternal caloric reserves (12, p. 496). Lusk asserts (13, p. 529) that lactation does not appear to increase the heat production.

This is not strange [he writes] since the rearrangement of food materials in the preparation of milk depends on hydrolytic cleavages and syntheses which involve hardly any thermal reactions, and also because it is known that the secretory activity of a gland has no influence upon the total heat production of the body.

The calories contained in the excreta constitute an additional energy loss to the maternal body. The approximate amount of this decrement has been determined by the use of the following factors described by Lusk (13, p. 53) and by Murlin and associates (30): each gram of urinary nitrogen was considered to represent the loss of 7.9 calories and each gram of dry feces was taken as representing the loss of 6.2 calories. By this method of calculation the average output in the urine of subjects VI, VII, and VIII was found to be 140, 125, and 130 calories, respectively, per day, and the daily loss in the feces 254, 330, and 270 calories, respectively. These figures probably underestimate this caloric loss.

The difference in the caloric intake in food and the outgo in breast milk and the excreta constitutes a crude approximation of the surplus available for maintenance and the dissipation of energy in the processes of milk secretion. The principal determinate factors in the maintenance energy requirement are the basal metabolism and muscular activity. The basal metabolic needs of women have been studied in detail and can be approximated for these subjects as 1300 to 1500 calories per day. Since the exact energy requirement for muscular activity for these women who were doing their own housework, tending their children, and leading their customary social lives could not be determined, their maintenance needs can be approximated only within the wide range of 2200 to 3200 calories a day.

Beyond the calories lost to the mother in the breast milk and excreta, an allowance must be made for the dissipation of energy in the physiological processes of milk secretion. In this connection, Rose (31, p. 118) has suggested that in planning a dietary for the lactating woman an allowance equal to 10 per cent of the caloric output in milk should be made. Rand, Sweeny, and Vincent (32, p. 217) state that in addition to the maintenance requirement, the provision of two food calories for each calorie secreted in breast milk is desirable. If the maternal organism must sacrifice an amount equal to 10 per cent of the caloric output in milk, then 2300, 1600, and 1200 calories daily would have been used up by subjects VI, VII, and VIII, respectively, for that purpose, whereas the dissipation of a quantity equal to the caloric content of the milk would have required the use of 4200, 3000, and 2200 calories, respectively, beyond maintenance. Consequently, if these mothers were 90 per cent efficient in the transformation of food energy into milk energy, as assumed by Rose, 2000, 3000, and 2600 calories daily for subjects VI, VII, and VIII, respectively, would be available for maintenance; on the other hand, if they were only 50 per cent efficient, as assumed in the second dietary regimen, then 100, 1600, and 1600 calories daily, respectively, would have been available for maintenance. From the

above summary it would appear that the women of this investigation exhibited a high degree of efficiency in the transformation of food energy into milk. Subjects VI and VII, producing the largest quantities of milk (3134 and 2366 cc. respectively), lost 7.6 and 3.2 kilograms respectively in body weight during lactation, but this total loss in body weight extending over a 60-week period could not have been a conspicuous source of calories for physiological activities. The caloric overfeeding which would have prevailed if these women had taken the overly generous supply of two food calories for each breast milk calorie beyond maintenance requirements, a practice indicated in the dairy industry, has not been demonstrated to be advantageous either to the cow (33, p. 130; 34, p. 514) or to woman, and indeed may prove inimical to milk flow in woman (28, 35).

These findings on three women who are physiologically endowed with the ability to secrete from two to three times the amount of milk ordinarily produced by women, show that it would have been undesirable to follow the dictum of animal experiments in providing for their body needs. Differences in species, such as exist between the ruminant cow and woman, have to be considered in the physiological utilization of food calories in the secretion of milk and in the appraisal of lactation requirements. The results here recorded suggest an urgent need for more comprehensive observations on women and a closer scrutiny of the present empiric standards for lactation, which are based largely upon experiments on mammals other than man.

*Fat.* As would be expected, the concentration of fat in breast milk resembled the caloric concentration. The highest fat concentration was achieved by subject VIII, who produced the smallest volume of milk. The milk volume ratios for subjects VI, VII, and VIII were 15:12:7, and the corresponding milk fat values per liter were 40, 36, and 48 grams, respectively. It is a common observation in the dairy industry that the amount of milk fat tends to vary inversely with the volume output. Subject VII chose slightly less dietary fat and much more carbohydrate than the other two women, but she did not produce as rich a milk as they.

In the experiments reported here, milk fat represented an even greater percentage of the food fat (Table II) than was observed in the caloric exchange. Subjects VI, VII, and VIII secreted 128, 84, and 73 grams of fat into breast milk in a 24-hour period on food containing respectively 194, 167, and 170 grams of fat. Thus milk fat was equivalent to 74, 50, and 43 per cent of the total intake in these respective cases. The quantity of fat remaining for utilization in metabolism amounted to 0.9, 1.2, and 1.3 grams per kilogram of body weight, which can be regarded as an adequate

but not liberal allowance. In connection with fat consumption, Rose (26, p. 25) states that "The average man's maximum capacity is said to be about 200 grams per day and he does not seem to maintain the best health with less than about 75 grams per day."

TABLE II  
AVERAGE DAILY FOOD INTAKE AND BREAST MILK OUTPUT

Subjects	Proteins			Fats			Carbohydrates		
	VI	VII	VIII	VI	VII	VIII	VI	VII	VIII
Food, grams.....	160	165	150	194	167	170	488	600	425
Breast milk, grams.....	33	25	18	128	84	73	222	163	99
Surplus, grams.....	128	141	133	60	76	100	268	437	324
Surplus, per kilogram body weight...	2.1	2.2	1.7	0.9	1.2	1.3	4.0	6.8	4.1
Breast milk in percentage of food intake.....	21	15	12	74	50	43	47	28	24
Breast milk grams per liter.....	10	10	12	40	36	48	70	70	68

Meigs (18) states that there is little doubt that amino acids, phosphatids, and glucose "are really the general currency of metabolism—the chemical forms in which the proteins, fats, and carbohydrates are distributed by the blood to all of the organs and tissues of the body." Meigs reviews both the experiments indicating that the phosphatids are the direct precursors of milk fat and the conflicting data of Foa (36), who contends that milk fat is formed from the triglycerides of blood. Regardless of the precise precursor of milk fat, it appears that under ordinary circumstances it is formed from food lipids, and hence is influenced in composition by the character of the diet (37). If the dietary fat is inadequate, food carbohydrates, proteins, and deposit or tissue fat will be used as a source of milk fat. The experiments of Maynard and McCay (37) demonstrated that the fat secreted by the milking cow and goat on a low-fat ration had a lower iodine number than did that produced on a normal ration.

Fat is the most variable constituent of milk. Several factors, including the level of dietary fat or protein and the milk volume, may affect it quantitatively and qualitatively. The literature reviewing the influence of dietary fat on the quantitative secretion of milk fat shows seemingly contradictory and inconclusive results.

*Carbohydrate.* In accordance with similar observations on dairy animals, little difference was found in the concentration of lactose in the milks of the women of the present study, although there were individual variations in milk yield and in carbohydrate consumption. The lactose values per

liter of breast milk were approximately 70 grams for all three women. Subjects VI, VII, and VIII chose average daily diets containing respectively 488, 600, and 425 grams of carbohydrate, and were able to secrete 222, 163, and 99 grams of lactose into their milk. These quantities of lactose were equivalent to 47, 28, and 24 per cent of their respective carbohydrate intakes. The surplus of food carbohydrates over lactose secreted amounted to 4.0, 6.8, and 4.1 grams per kilogram of body weight for subjects VI, VII, and VIII, respectively.

There is a great deal of evidence to show that lactose is derived from blood sugar. Observations on lactating animals have shown that lactose is the most constant of the main organic constituents of milk and that unlike proteins and fats its concentration is not easily affected by changes in the ration.

*Protein.* According to Sherman (38), an allowance of 1.0 gram of protein per kilogram of body weight provides liberally for adult maintenance. The surplus food protein beyond that given out in breast milk by subjects VI, VII, and VIII amounted respectively to 2.1, 2.2, and 1.7 grams per kilogram of body weight. There was thus a considerable amount of protein for dissipation in milk secretion. Rose (39, p. 452) and McLester (40, p. 273) state that the human mother is only about 60 per cent efficient in the secretion of milk protein and that, accordingly, in lactation two grams of dietary protein, in addition to the maintenance intake, should be ingested for each gram of protein in breast milk, or 0.75 grams of additional protein for each ounce of milk produced. After deducting the amount of protein used in milk secretion, according to the above proposals, subjects VI, VII, and VIII would have had respectively 94, 115, and 114 grams of protein left for the remaining body functions.

Subjects VI, VII, and VIII chose liberal diets which contained an average of 160, 165, and 150 grams of protein per day, respectively (Table II). They secreted 3134, 2366, and 1419 cc. of milk daily, containing 33, 25, and 18 grams of protein, representing 21, 15, and 12 per cent respectively of the protein intake. The excellent quality of the ingested protein is shown by the fact that 55 to 75 per cent of it was derived from animal sources, chiefly from milk, while cereals constituted the largest fraction of the vegetable portion.

The concentration of protein in breast milk was similar for all subjects (10 to 12 grams per liter), but again the milk of the smallest producer, subject VIII, showed a 20 per cent higher value than that of the other women.

It is difficult to determine the protein requirement of an individual, since it depends upon the amino acid content of the assimilated protein,

the number of non-protein calories, and the composite specific amino acid needs of all body tissues under the particular conditions existing at the time. It is quite probable that the amino acid requirements for special body functions such as growth, maintenance, or lactation are different in quantity and quality, so that the efficiency of protein utilization in each case will depend on the specific demands of the tissues and the particular proteins involved. In addition, the effectiveness of the preparation<sup>7</sup> for lactation during pregnancy constitutes a decisive factor in the success of milk secretion. Because of the multiplicity of determinate influences, it becomes difficult to set a physiological minimum for any body function and, indeed, it is impossible to state a quantitative protein requirement for complicated physiological functions such as those involved in lactation superimposed on maintenance. On the other hand, the provision of an adequate protein intake during lactation is of importance, since it has been shown that milk protein is ultimately derived from food protein and that the level of dietary protein affects the quantity of milk secreted and, within limits, its composition (18, 19).

The milk proteins are derived from the free amino acids of the blood. It has been shown that the quantity and quality of the amino acids in the circulating blood are changed by diet, by the varying nitrogen demand of the tissues, and by the supply of non-protein calories. These changes have a marked influence on milk secretion and, in addition, protein metabolism is intimately related to the metabolism of carbohydrate and fat. Meigs (18) states that

A change in the carbohydrate of the diet may affect the amino-acids of the blood, and thereby the secretion of milk protein; and a change in the protein of the diet may affect the secretion of milk fat—probably again through a change in the amino-acids of the blood.

The women of this study were successful milk producers. Their daily dietaries during pregnancy were liberal in calories and included 90 to 133 grams of protein of high biological value. All but five of the twenty-five nitrogen metabolic balances during pregnancy and lactation were positive. There is evidence to indicate that nitrogen retention beyond that required by the fetus and adnexa favors success in lactation (41 to 44). During the period of increased maternal demands, these subjects ingested larger quantities of food, including protein of excellent quality.

*Calcium and Phosphorus.* In accordance with findings from experimental work on animals, the calcium and phosphorus values of the breast milk for the three women of these studies were found to be fairly constant. The intake and outgo of these substances are summarized in Table III, where it is shown that the calcium in breast milk amounted to 1.17, 0.75, and

0.45 grams daily for subjects VI, VII, and VIII, respectively, and was equivalent to 41, 27, and 15 per cent of the total calcium intake of 2.71, 2.82, and 3.08 grams daily. The calcium intake stood in inverse relation to milk volume and hence to the total excretion in breast milk.

TABLE III  
AVERAGE DAILY MINERAL INTAKE IN FOOD AND OUTGO IN BREAST MILK

Subjects	Calcium			Phosphorus		
	VI	VII	VIII	VI	VII	VIII
Food, grams.....	2.71	2.82	3.08	3.15	3.31	3.28
Breast milk, grams... ..	1.17	0.75	0.45	0.36	0.35	0.20
Surplus, grams .....	1.54	2.08	2.63	2.78	2.96	3.08
Surplus per kilogram body weight..	0.023	0.033	0.033	0.041	0.046	0.038
Milk content as percentage of intake	41	27	15	11	11	6
Milk value, grams per liter.....	0.36	0.32	0.32	0.12	0.16	0.14

Similarly the phosphorus outgo in milk of 0.36, 0.35, and 0.20 grams per day was equivalent to 11, 11, and 6 per cent of the phosphorus intake of 3.15, 3.31, and 3.28 grams per day for subjects VI, VII, and VIII, respectively. The phosphorus intakes were likewise in inverse relation to the milk yield and to phosphorus outgo in breast milk.

Sherman (38) found the adult minimum maintenance requirements to be 0.0064 grams of calcium and 0.0126 grams of phosphorus per kilogram of body weight. In order to insure a factor of safety he suggested that 50 per cent more of each element, or 0.0096 grams of calcium and 0.0189 grams of phosphorus per kilogram of body weight, be regarded as an adequate allowance for these substances. The surplus of calcium intake over calcium outgo in milk was 0.023, 0.033, and 0.033 grams per kilogram of body weight. The phosphorus intake exceeded the phosphorus outgo in the milk by 0.041, 0.046, and 0.038 grams per kilogram of body weight for subjects VI, VII, and VIII respectively.

Although their diets contained liberal quantities of calcium and phosphorus, chiefly from foods such as milk, eggs, and cheese, from which these elements are usually well assimilated, the women of this investigation were unable adequately to absorb or utilize calcium and phosphorus while maintaining a high level of milk flow. Only one of thirteen metabolic calcium balances during lactation was positive, while four of thirteen metabolic phosphorus balances during this period were negative (16, 17). The negative calcium balances were largely due to the excessive excretion of that element in the feces. In three of thirteen balances the fecal excretion of cal-



cium exceeded the intake, and in eight of thirteen the fecal excretion was more than 75 per cent of the intake.

The consideration of the dietary calcium and phosphorus intake and the outgo of these substances in milk is particularly pertinent with reference to the subjects of this study, who were secreting large quantities of milk and were found to have negative calcium and phosphorus balances while maintaining this high level of milk flow (17). Meigs and Cary (12, p. 511) state that studies of calcium and phosphorus metabolism have shown that

the bones serve as a great storehouse for these elements, which can store up a surplus in periods of plenty and yield it up again in periods of need. And again,

If either calcium or phosphorus is deficient in the food of a milking animal, she will take both elements from her bones and use whichever one is necessary to keep up the normal composition of the milk.

From these data on the nutritive performance of three women during lactation it is evident that providing an adequate diet is not the only essential; more satisfactory ways and means of inducing better digestion, absorption, and assimilation of the food materials taken into the body must be devised if lactation is to leave the maternal body unimpaired.

#### SUMMARY

A quantitative study of the self-chosen diets of three multiparae is reported. The nutritive requirements during 60 weeks of intensive milk flow are considered, with particular reference to the relationship between the intake of food calories, proteins, fat, carbohydrates, calcium, and phosphorus and the outgo of these components in breast milk.

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## THE IRON REQUIREMENT OF THE PRE-SCHOOL CHILD\*

By

JANE M. LEICHSENRING AND IVA HANSEN FLOR  
(*From the Division of Home Economics, University of  
Minnesota, University Farm, St. Paul.*)

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AT THE time this study was undertaken, no information on the iron requirement of young children based on balance experiments was available. Since then there has appeared the report of an iron-balance study by Rose, Vahlteich, Robb, and Bloomfield (4) on a girl 31 months of age.

The present investigation was carried out as a typical balance experiment in which the utilization of iron at two levels of intake was compared. Four healthy pre-school children, one boy and three girls, whose ages ranged from 35 to 56 months, served as subjects. The experiment was divided into two periods of 5 days each. In period I a diet low in iron was given and in period II a diet high in iron was supplied. All food consumed by these children over the 10-day period of the experiment was weighed and prepared by one of us. Splendid coöperation was obtained from the parents who understood and appreciated the precautions necessary in order to make the investigation a success.

On the basis of the figures in Sherman's tables (5), the diet in period I was calculated to contain 5 milligrams of iron and the diet in period II, 8.5 milligrams. The results of the analyses of the various foods included in the diet showed, however, that the iron intake actually fell considerably below this estimated amount, the analyses showing 3.25 milligrams and 6.5 milligrams respectively for the 2 diets, or 35 per cent and 24 per cent less than the calculated amounts. This observation is in accord with that of Rose, Vahlteich, Robb and Bloomfield who report that the iron content of their diet, as calculated from Sherman's tables, was over 30 per cent higher than the actual amount as determined by analyses. In the present study all foods which contributed significant amounts of iron to the diet were analyzed. The results of these analyses appear in Table I.

The diet during the period of low iron intake consisted of orange juice, puffed rice, egg, potato (peeled), white bread, canned peaches, canned tomatoes, canned string beans, rice, milk, cream, butter, and sugar. Dur-

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ing the period of high iron intake the diet included: raisins, shredded wheat biscuit, white and graham bread, chopped beef, potato (peeled), carrots, prunes, canned peas, lettuce, egg, milk, cream, butter, sugar, and cornstarch. The variety and combination of foods was such that no difficulty was experienced in getting the children to eat the same foods for the 5-day period. Distilled water was allowed in unlimited quantities for drinking purposes and was also used in the food preparation. The diet, as planned, was based on the accepted dietary standards for children.

TABLE I  
IRON CONTENT OF FOOD MATERIALS PER 100 GRAMS EDIBLE MATERIAL

	Analysis 1 mgm.	Analysis 2* mgm.
Bread, graham.....	1.19	—
Bread, white.....	0.67	—
Carrots.....	0.39	0.63
Egg, cooked.....	1.92	—
Lettuce.....	0.31	0.56
Meat, beef, round.....	3.45	3.02
Milk, whole.....	0.18	—
Orange juice.....	0.14	0.24
Peaches, canned.....	0.25	—
Peas, canned.....	1.23	—
Potatoes, peeled.....	0.39	0.52
Prunes.....	2.33	1.72
Raisins.....	4.04	2.43
Shredded wheat biscuit.....	3.05	—
String beans, canned.....	0.38	—
Tomatoes, canned.....	0.16	—

\* Where no figure appears in this column, the same analysis covers both experimental periods.

The first two days on each of the experimental diets were used as a preliminary period to allow for an adjustment of the iron balance to this level of intake. During the following 3 days, collections of urine and feces were made. The urine samples were measured and stored in sealed glass bottles, using toluol as a preservative. For the marking of the feces, a 0.2 gram carmine capsule was given at the beginning and end of the collection period. The feces were covered with a mixture of concentrated hydrochloric acid and 95 per cent alcohol (1:3) and dried on the steam bath in a covered evaporating dish. When dry the feces were weighed, ground in a mortar, thoroughly mixed, and stored in sealed glass bottles.

All foods which have good keeping qualities were purchased in one lot for the entire experiment. Every effort was made to have the foods which

were purchased from day to day as nearly uniform in composition as possible. Samples for analyses were always taken at the time the foods were weighed for the diet. The composite food samples thus obtained were dried in covered evaporating dishes. The air-dried food was weighed, ground in a mortar, and, after thorough mixing, was stored in sealed glass bottles.

Analyses of food, feces, and urine for iron were made in triplicate using the potassium permanganate titration method as modified by Murray (3). As a preliminary preparation of the urine, a modification of the Wolter method (Hawk and Bergeim, (1)) was used. Three cubic centimeters of concentrated nitric acid were added to a 150 cc. sample of urine. This was allowed to evaporate to a low volume on the steam bath. The residue was then transferred to a crucible and charred over a very low flame. Ashing was completed in the muffle furnace at 500°C. The size of the samples of food and feces used for analyses was such as to insure approximately 0.2 milligram of iron in each sample. Correction was made for the iron content of all reagents used. Every precaution was taken to avoid iron contamination of materials by keeping them constantly covered during storage, preparation, and analytical procedures and by using only such utensils as contain no iron.

### DISCUSSION

The average daily excretion of iron in the urine and feces of the four subjects is shown in Table II. It will be noted that there was a considerable difference in the amount of urinary iron excreted by the 4 subjects and also that there was a distinct increase in the amount of iron in the urine during the period of high iron intake in three of the subjects. The average amount of urinary iron reported by Rose, Vahlteich, Robb, and Bloomfield for the subject in their experiment was 0.20 milligram on an iron intake of 4.64 milligrams; a figure which is intermediate when compared with the four subjects of this experiment on the low-iron diet, but low when compared with the figures in this study on the high-iron diet. The daily fecal output of iron during the period of low-iron intake was fairly uniform for the four subjects in this study, the range being from 1.34 to 2.00 milligrams per day. Greater variation was observed during the period of high-iron intake, the range being 2.07 to 3.21 milligrams. The total daily output on iron averaged 2.07 milligrams during the period of low iron intake, and 3.29 milligrams during the period of high-iron intake. All of the children were in positive balance throughout. The average daily iron retention during the period of high-iron intake was 2.02 milligrams greater

than during the period of low-iron intake, or an increase of 170 per cent over the amount retained during the period of low-iron intake.

Obviously, the iron intake in period I did not meet the needs of these children for optimal storage of this element, since a much greater amount was retained during the period of high-iron intake. If the level of iron excretion in period I may be taken as an index of the maintenance requirement of these children, it would appear that approximately 2.1 milligrams

TABLE II  
AVERAGE DAILY INTAKE AND OUTPUT OF IRON  
PERIOD I—LOW IRON INTAKE

Subject	Sex	Age	Energy intake	Iron intake	Iron output in urine	Iron output in feces	Total iron output	Iron retention
		mo.	cal.	mgm.	mgm.	mgm.	mgm.	mgm.
A	Male	55	1280	3.12	0.11	1.74	1.85	1.27
B	Female	35	1006	2.74	0.13	1.34	1.47	1.27
C	Female	46	1349	3.52	0.48	2.00	2.48	1.04
D	Female	56	1429	3.64	0.58	1.90	2.48	1.16
Average		48	1266	3.25	0.30	1.75	2.07	1.19

PERIOD II—HIGH IRON INTAKE

A	Male	55	1352	6.61	0.22	2.07	2.29	4.32
B	Female	35	1296	6.47	0.30	2.93	3.23	3.24
C	Female	46	1426	6.42	0.81	3.09	3.90	2.53
D	Female	56	1444	6.50	0.53	3.21	3.74	2.76
Average		48	1380	6.50	0.49	2.80	3.29	3.21

of iron are needed daily for this purpose or, on the basis of body weight, the requirement would be 0.12 milligram per kilogram. Calculating the maintenance needs of children of this age on the basis of the adult requirement of 10 milligrams iron per 70 kilograms body weight (Sherman, 5) would give a maintenance requirement of 2.4 milligrams per day. This figure agrees so closely with the maintenance requirement of 2.1 milligrams observed in this study as to suggest that on the basis of body weight, the maintenance need of the child is similar to that of the adult. The maintenance requirement observed by Rose, Vahlteich, Robb, and Bloomfield on their subject was 5.70 milligrams per day or 0.41 milligram per kilogram, a requirement which is very much higher than that of any of the subjects in this study (Table III).

Inasmuch as the excretion of iron during period II was considerably above the level of excretion in period I, it would seem that this higher level of intake more than supplied the needs for optimal storage. It is recognized, of course, that a part of this increase may result from a failure of absorption of iron due to its unavailability and to that extent it does not represent a surplus. Assuming, however, that optimal storage occurred during this period of high-iron intake, it would appear from the results of

TABLE III  
IRON REQUIREMENT FOR MAINTENANCE AND GROWTH  
BASED ON DATA IN THIS STUDY

Subject	Energy intake	Weight	Iron Requirement				
			For maintenance	For growth	Total requirement for maintenance and growth	Total requirement per kg. of body weight	Total requirement per 100 Cal. of food intake
	cal.	kgn.	mgm.	mgm.	mgm.	mgm.	mgm.
A	1316	16.40	1.85	4.32	6.17	0.377	0.475
B	1151	15.19	1.47	3.24	4.71	0.310	0.270
C	1387	18.60	2.48	2.52	5.00	0.274	0.361
D	1436	18.49	2.48	2.76	5.24	0.283	0.365
Average	1322	17.15	2.07	3.21	5.28	0.308	0.392

this study that the iron requirement for growth was approximately 0.2 milligram per kilogram. The iron requirement for maintenance and for growth of the four children based on the above assumptions is shown in Table III. The calorie intake is the average for each child over the two experimental periods.

On the basis of the findings in this study, the following iron allowances for pre-school children may be tentatively suggested: for maintenance, 0.12 milligram per kilogram; for growth, 0.20 milligram per kilogram; or a total of 0.32 milligram per kilogram. Since the results of the food analyses in this experiment indicate that the amount of iron in different foods may be considerably less than the most commonly used figures, and also since there are pronounced individual differences in the iron requirement of children, it would seem that a margin of safety of at least 50 per cent above this calculated requirement of 0.32 milligram per kilogram should be allowed in planning the dietaries of pre-school children. This would make a standard allowance of 0.48 milligram per kilogram. On this basis



the total daily iron requirement of the children in this study would be 8.23 milligrams or 0.62 milligram per 100 calories. These figures are in close agreement with the allowance of 8.5 milligrams or 0.76 milligram per 100 calories suggested by Rose, Vahlteich, Robb, and Bloomfield, and also with the iron intake of children in private homes observed by McKay (2). She reports an average of 8.17 milligrams of iron in the diets of pre-school children in private homes as compared with 4.37 milligrams in the diets of children in an institution.

### SUMMARY

1. Two iron-balance studies were conducted using four healthy children, one boy and three girls, ranging in age from 35 to 56 months. Iron retentions at two levels of intake were compared.

2. The results of the food analyses indicate that the iron content of foods may show considerable variation from the most commonly used figures. Diets which were planned to contain 5 and 8.5 milligrams of iron actually contained only 3.25 and 6.5 milligrams respectively.

3. On a diet containing 3.25 milligrams of iron, an average of 1.2 milligrams was retained daily, whereas, on a diet containing 6.5 milligrams of iron, 3.2 milligrams, or nearly three times as much as during the period of lower iron intake, were retained.

4. The observed maintenance need of the children in this study was approximately 0.12 milligram per kilogram. It would appear from these results that, on the basis of body weight, the maintenance requirement of the child is similar to that of the adult.

5. The iron requirement for growth, as observed in this study, was approximately 0.2 milligram per kilogram.

6. In view of the differences observed between the actual iron content of the food used and the amounts as calculated from Sherman's tables, and also the pronounced individual differences in the iron requirement of children, it is suggested that a 50 per cent margin of safety be allowed above this observed total requirement of 0.32 milligram per kilogram. This would make a standard allowance for children of this age of 0.48 milligram per kilogram, or 0.62 milligram per 100 calories, or a total of 8.23 milligrams per day.

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## DIETARY REQUIREMENTS FOR FERTILITY AND LACTATION

### XXIV. FURTHER STUDIES ON THE SPECIFIC EFFECT OF VITAMIN B ON LACTATION\*

By

BARNETT SURE AND MARGARET ELIZABETH SMITH

*(From the Departments of Agricultural Chemistry and Home  
Economics, University of Arkansas, Fayetteville.)*

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**I**N a recent communication it was demonstrated that when albino rats are transferred on the day of birth of their litters from our stock diet to a ration deficient only in vitamin B, vitamin G having been furnished in abundance by the introduction of 10 per cent autoclaved Northwestern yeast in the ration, infant mortality to the extent of 70 to 100 per cent was encountered in one to two weeks. When, however, litter mate lactating animals were restricted to the same daily food and water intake of the same diet but which contained 10 per cent untreated Northwestern yeast, thus providing adequate amounts of both vitamins B and G, there was 80 to 100 per cent survival of young, and growth of litters actually took place but at a subnormal rate. It, therefore, became apparent that vitamin B, in addition to stimulating the appetite, exerts a specific beneficial influence on lactation unrelated to food intake.

In this study additional experimental evidence was secured which corroborates the previous findings of Sure and Walker (1). A few experiments were conducted in pairs, the majority, however, having been carried out in triads. The object was to produce vitamin B deficiency in the lactating rats and nursing young gradually during the middle or later part of lactation, aiming particularly at prolonged maintenance, so that such baby rats could be used for blood studies in lipid metabolism without the possible disturbance of the complicating factor of catabolism of body tissue. The modified technic was carried out as follows:

Lactating rats were transferred on the day of the birth of their litters, reduced to 6 in number, from stock diet No. 1 (2) to Ration 1865, satisfactory in every respect with the exception of vitamin B, of the following composition: Casein,<sup>1</sup> 20; salt mixture, 185 (3), 4; autoclaved yeast,<sup>2</sup> 10;

\* Research paper No. 203, Journal series, University of Arkansas.

<sup>1</sup> Purified by extraction for 10 days with acidulated water.

<sup>2</sup> Northwestern dehydrated yeast was autoclaved in shallow glass Pyrex dishes for 6 hours at 20 pounds pressure.

butter fat, 10; dextrin, 56. Litter mates were restricted to the same amount of water and food of the same diet, but the yeast contained in the ration was untreated. The only limiting factor on such a dietary regime, since the plane of nutrition was controlled, was vitamin B destroyed during the process of autoclaving. Another group of litter mates was fed the same ration containing 10 per cent of the untreated yeast but the daily food and water intake was unrestricted. The difference between the second and third groups must then be due to inanition, since the only limiting factor was the plane of nutrition. In order to prevent early infant mortality and produce prolonged maintenance of the young, the mother on the vitamin B-deficient ration was given from the day of delivery of her litter a supplementary allowance of 500 mg. of Northwestern yeast separately from the ration. Such an administration of yeast was also given to the lactating rats of the second and third groups, so as to keep all the dietary factors constant. After the pathological litters had reached a collective weight of 110 to 120 gms., the yeast was either gradually or entirely removed from the mothers as well as from the control animals. The subsequent slow growth in the pathological nursing young can be explained by the securing of stored vitamin B from the mothers. In a few cases when it was anticipated, after the young began eating, that traces of vitamin B may have been furnished by the yeast in the ration, which was autoclaved for 6 hours at 20 pounds pressure, the yeast was replaced by another batch autoclaved for 8 hours at 25 pounds pressure according to a recent suggestion by Salmon (4).<sup>3</sup>

From our numerous records we have selected four typical cases, one pair and three triads, which are shown graphically in Charts 1 to 4 inclusive.

An examination of curves 1 and 2 clearly shows that up to the 9th day no difference is evident in the lactation efficiency index produced by vitamin B in the diet. However, from then on the effect of vitamin B *per se* becomes more and more pronounced, so that on the 36th day the pathological litter on the maternal diet, deficient in vitamin B, weighed 120 gm. and the control litter, on the same maternal diet, but containing an abundance of vitamin B, and restricted to the same daily food and water intake, weighed 180 gm. On the 46th day the pathological litter was reduced to 3 which were in a dying condition, while in the control litter there were 5 young left on that day in an apparently healthy, vigorous condition.

<sup>3</sup> We have evidence that the yeast under this more drastic treatment at its natural pH supplied an abundance of the vitamin G growth-promoting factor.

The specific influence of vitamin B on lactation is very marked, as designated by curves 1 and 2. For a period of 23 days while the pathological litter had practically maintained its weight, the control litter, whose maternal diet on the same plane of nutrition contained vitamin B, gained 70 gm. The specific effect of vitamin B on lactation at the termination

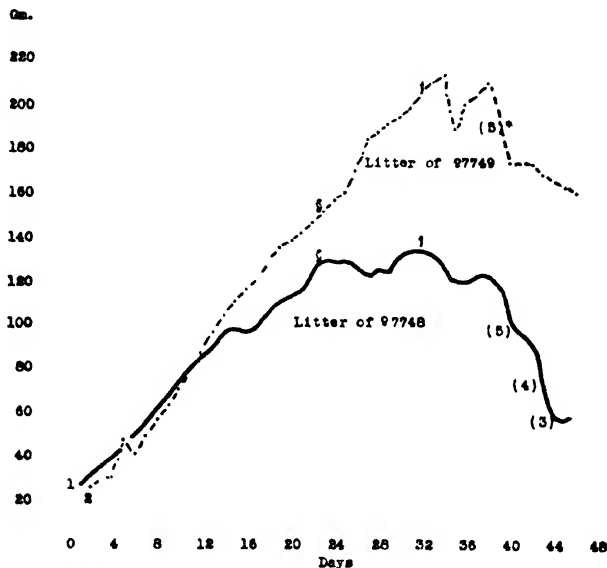


CHART 1. Curve 1 represents the lactation record of ♀ 7748, the diet of which was satisfactory in every respect but deficient in vitamin B, 10 per cent autoclaved Northwestern dried yeast serving as the source of vitamin G; Curve 2, the lactation record of ♀ 7749, on the same diet containing 10 per cent untreated Northwestern yeast, and, therefore, supplying an abundance of both vitamins B and G. Since ♀ 7749 was restricted to the same daily food and water intake as that consumed by its litter mate, ♀ 7748, the limiting factor in the diet of lactating rat 7748 was vitamin B. Since it was desired to furnish to lactating ♀ 7748 a diet inadequate rather than entirely deficient in vitamin B, she was given from the day of the birth of her litter a daily supplement, separately from the ration, of 500 mg. Northwestern yeast which was also furnished at the same time to ♀ 7749. At point "§" the yeast was reduced to 300 mg. daily and at point "†" the administration of yeast was discontinued. Figures in parentheses represent the number of young left on that particular day. Keeping the plane of nutrition constant, the specific effect of vitamin B on lactation becomes apparent as is demonstrated in this and the succeeding charts.

\* One young was accidentally killed in closing door of cage, leaving 5 in the litter.

of the experiment is expressed by the difference in the collective weights of litters of ♀ 7788 and of ♀ 7787, which is 240 gm. - 153 gm. = 87 gm. The inanition effect is expressed by the difference in lactation performance of ♀ 7788, which was restricted, and ♀ 7789, which was unrestricted on the same complete diet. Although ♀ 7788 was actually able to wean her litter of 5 young, she was able to do so only 8 days later than

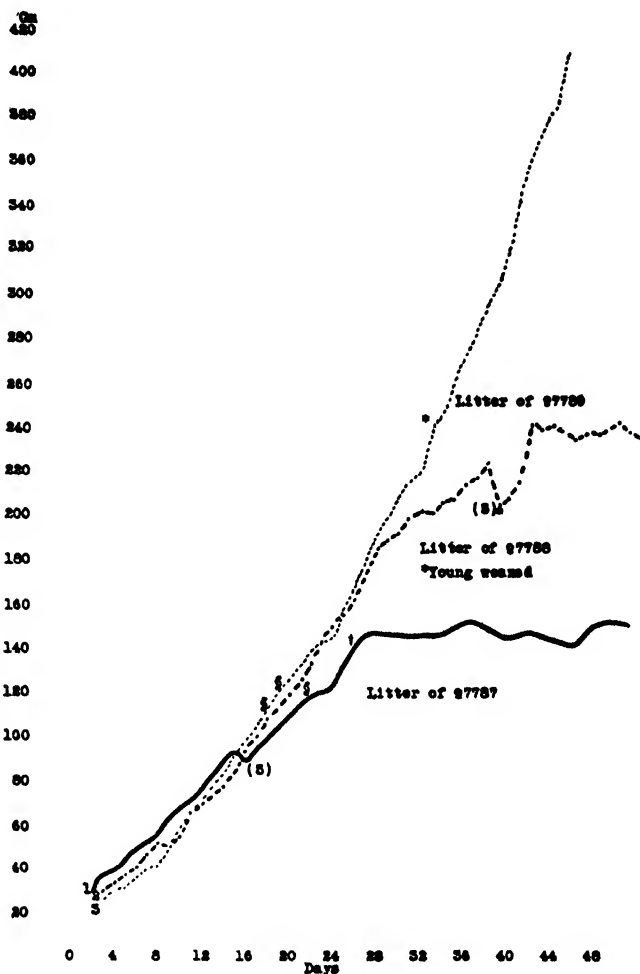


CHART 2. Curve 1 represents the lactation record of Q 7787, the diet of which was deficient only in vitamin B, 10 per cent Northwestern yeast autoclaved for 6 hours at 20 pounds pressure serving as the source of vitamin G; Curve 2, the lactation record of litter mate, Q 7788, on the same diet containing 10 per cent of the yeast untreated, and, therefore, furnishing an abundance of vitamins B and G; Curve 3, the lactation record of Q 7789 on the same daily food and water intake as its litter mate, Q 7788, but was unrestricted to its daily food and water intake. All the lactating rats received from the day of the birth of their litters 500 mg. daily of the Northwestern yeast separately from the ration. At point "§" the daily administration of the 500 mg. yeast was discontinued. At point "†" the autoclaved yeast in the ration of Q 7787, which was heated for 6 hours at 20 pounds pressure, was replaced by a batch autoclaved for 8 hours at 25 pounds pressure. The latter insured the absence of any traces of undestroyed vitamin B but still provided an adequacy of vitamin G for the requirements of nursing young.

Figures in parentheses represent the number of young left on that particular day.

The difference in lactation performance between females 7787 and 7788 is due to the specific effect of vitamin B. The difference in lactation efficiency between females 7788 and 7789, however, is due to the difference in the plane of nutrition.

her litter mate, ♀ 7789, on the same unrestricted diet. The difference in the collective weight of the litters at weaning time was 39 gm. The difference after weaning is much more accentuated as shown by curves 2 and 3.

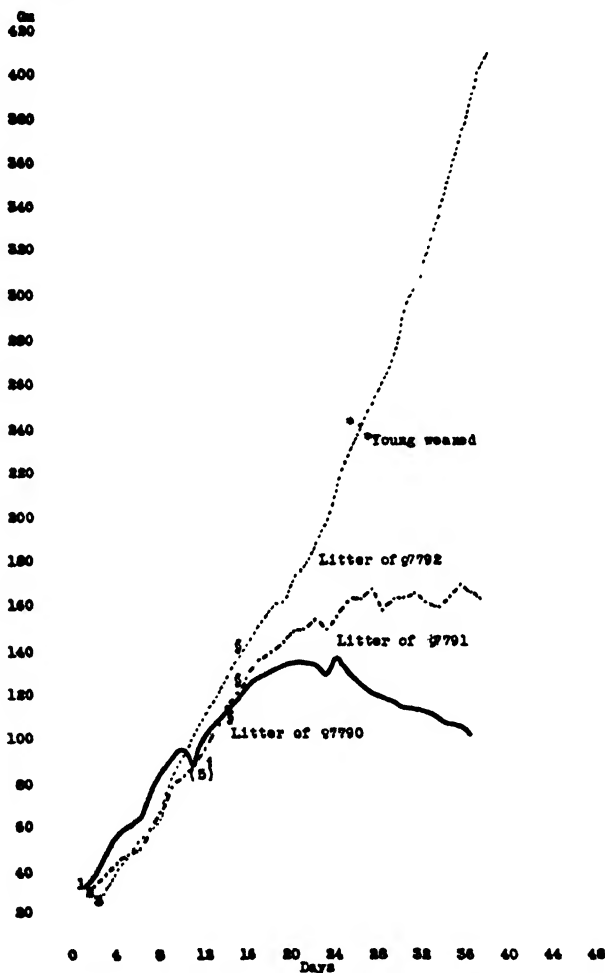


CHART 3. Curve 1 represents the lactation record of ♀ 7790 on a diet deficient only in vitamin B; Curve 2, the lactation record of a litter mate, ♀ 7791, on the same diet supplemented with an abundance of vitamin B, but restricted to the same plane of nutrition as ♀ 7790; Curve 3, the lactation record of litter mate, ♀ 7792, on the same diet as ♀ 7791 but unrestricted as to its daily food and water intake. From the day of the birth of their litters all these lactating rats received a daily supplement of 500 mg. of Northwestern yeast. At point "§" the yeast was removed. Sign "†" indicates that one young died leaving 5 in the litter. At point "•" the litter of ♀ 7792 was weaned. The specific effect of vitamin B and the influence of under nutrition are quite marked.

The specific effect of vitamin B on lactation does not become apparent in this group until the 13th day. From then on, ♀ 7790, on the vitamin B

deficient ration, lost one young and subsequently the litter of the female continuously lost weight until the 36th day when the experiment was terminated. The specific effect is expressed by the difference in the collective weight of litters of ♀ 7790 and ♀ 7791 (curves 1 and 2), which is 60 gm.

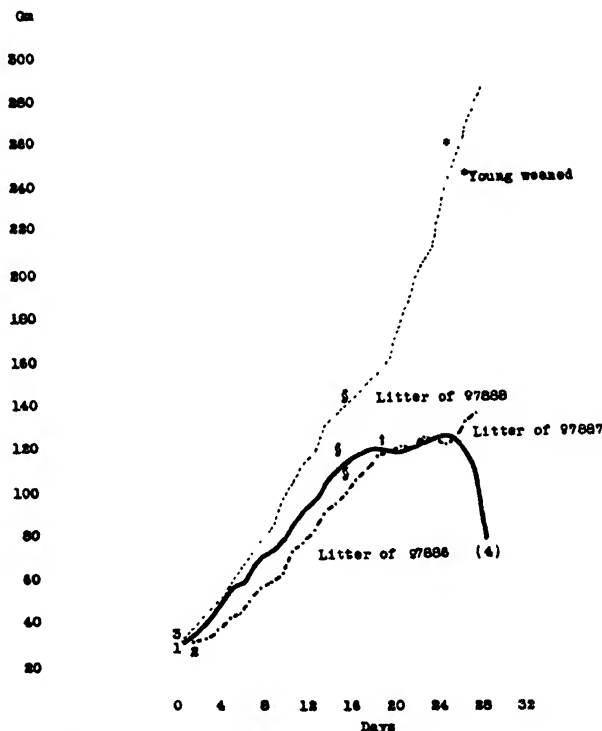


CHART 4. Curve 1 represents the lactation record of ♀ 7886 on a ration deficient only in vitamin B; Curve 2, the lactation record of litter mate, ♀ 7887, on the same diet fortified with an abundance of vitamin B but restricted to the same plane of nutrition as ♀ 7886; Curve 3, the lactation record of litter mate, ♀ 7888, on the same diet as ♀ 7887 but unrestricted to its daily food and water intake. All these nursing mothers received from the day of the birth of their litters a supplement of 500 mg. of Northwestern yeast which was discontinued at point "\$." Number in parentheses indicates that four young were left out of six. Two of the four remaining young were in a dying condition. At point "\*" the litter of ♀ 7888 was weaned. The specific effect of vitamin B on lactation and the influence of inanition on rearing of young are also quite pronounced.

The inanition effect at weaning is demonstrated by the difference in the collective weight of litters of ♀ 7791 and ♀ 7792 (curves 2 and 3), which is 80 gm. On the day the experiment was discontinued, the inanition effect was 240 gm.

In this particular group the specific influence of vitamin B on weaning of young did not become evident until the 29th day of lactation when

♀ 7886, on the vitamin B deficient ration, lost 2 young with 4 remaining young in a dying condition. On the same day the litter mate, ♀ 7887, was nursing all her 6 young which were in an apparently vigorous condition. The day the litter of ♀ 7888, on the unrestricted control diet, was weaned, there was an inanition effect to the extent of 120 gm. (curves 2 and 3).

In this connection, we would like to point out that very frequently we encounter what would seem as irregular lactation performance in case of litter mate controls receiving the vitamin B containing restricted diet. Occasionally for 7 to 10 days their litters show a poorer growth than the animals on the vitamin B deficient ration. This is explained as follows: In starting three litter mate females on days of delivery of their young on such studies, we frequently were compelled to take animals varying as much as 60 to 80 gm. in weight, and have found that the smaller animals, which were occasionally placed on the restricted diet, had considerably less vitamin reserves; and for that reason a delayed manifestation of the specific effect of vitamin B on lactation was frequently encountered.

#### SUMMARY

Further evidence is submitted that vitamin B, in addition to stimulating the appetite, exerts a specific influence on lactation, unrelated to the food and water intake.

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## THE SPECIFIC EFFECT OF VITAMIN B ON GROWTH\*

By

BARNETT SURE, M. C. KIK, AND MARGARET ELIZABETH SMITH

*(From the Departments of Agricultural Chemistry and Home  
Economics, University of Arkansas, Fayetteville.)*

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**I**N 1926 Drummond and Marrian (1) presented experimental evidence from which they drew conclusions that the physiological effects of vitamin B deficiency are essentially those of inanition or starvation. The lessened food intake, loss in weight, and fall in body temperature, they asserted, are all entirely the manifestations of inanition. The inference drawn, therefore, was that vitamin B produces growth only indirectly through stimulating the appetite. A careful analysis of the data of these investigators hardly justifies their sweeping conclusions as to the rôle of vitamin B in growth. In the first place they stated:

A series of rats were fed daily on a complete artificial diet, plus 5 per cent yeast extract, in amounts of the order that it was estimated would have been voluntarily consumed had the animals been given an ample supply of the vitamin B-deficient ration. Control groups provided with an unlimited supply of the complete diet and the deficient ration were also observed. The general result of these tests was that the groups fed on the restricted ration of the complete dietary (approximately 1 gm. daily per 10 gm. body weight) maintained both body weight and temperature, whilst occasional estimations of oxygen consumption yielded values within the normal range.

In the text of the article the statement is definitely made that, on the same daily food intake, animals receiving in addition vitamin B in the form of a yeast extract, did not show the symptomatology of inanition fever or loss of body weight; yet, in the summary of the paper these English investigators concluded that . . . "in the case of rats, at any rate, the nutritive failure following a deficiency of vitamin B is virtually identical with that of starvation." The additional experiments, from which Drummond and Marrian based their conclusions, were carried out as follows: To quote:

A number of parallel experiments to those just described were carried out in which animals of similar weight were given daily from 0.5–1 gm. of yeast extract as their sole food together with ample supplies of water. The amount of utilizable food, from the standpoint of calories, supplied in this yeast extract was negligible. In no essential did the behaviour of the animals in this group differ from those maintained on water alone. The provision of ample supplies of vitamin B neither prevented the final fall of body temperature, blood sugar and metabolism, nor prolonged life.

\* Research paper No. 204, Journal series, University of Arkansas.

Since the animals in both groups received no food, a specific effect of vitamin B on metabolism certainly could not have been ascertained.

In August of 1929<sup>1</sup> Rose, Stucky, and Cowgill (2) reported that in the majority of dogs studied, anhydremia was found to be a manifestation of a specific influence of a deficiency of the vitamin B complex unrelated to food intake.

Recently Drury, Harris, and Mandsley (3) have reported that deprivation of the vitamin B complex leads to a severe bradycardia in young rats, and that the slow heart rate is due not to the lowered food consumption but directly to the absence of the vitamin.

That vitamin B exerts a specific effect on lactation, unrelated to food intake, has been recently demonstrated by Sure and Walker (4) and by Sure and Smith (5).

In this communication we are reporting our results on the specific effect of vitamin B on growth of the non-lactating weaned albino rat. The first part of the investigation concerned itself with the vitamin B complex and the second part with uncomplicated vitamin B deficiency. About two hundred animals were employed in this study but to conserve space, only representative cases are submitted graphically in Charts 1 to 5 inclusive. The technic employed is essentially that described in the preceding communication and, therefore, needs no repetition here. The work on the vitamin B complex was carried out in pairs, while that on vitamin B was done in triads mainly.

Our findings, as illustrated in the accompanying charts, are self-explanatory and need little comment. An examination of Charts 1 and 2 clearly demonstrates that the vitamin B complex, as supplied by dehydrated Northwestern baker's yeast, produces a specific effect on growth unrelated to the plane of nutrition. Since, however, yeast contains an appreciable amount of foreign substances other than vitamin B, particularly minerals, the question arises whether such inorganic elements as copper (6) and manganese (7), recently reported to be essential in nutrition, may not have been complicating factors. That such is not the case is evident from our studies on uncomplicated vitamin B deficiency in which we used autoclaved yeast and the same yeast in the untreated condition at the same plane of intake. The only limiting factor was vitamin B, which was destroyed in the autoclaved yeast under our conditions of treatment, as is shown in the charted data.

In Charts 3 and 4 a differentiation is made between the specific effect of vitamin B and the higher plane of nutrition on growth.

<sup>1</sup> Published in February, 1930.

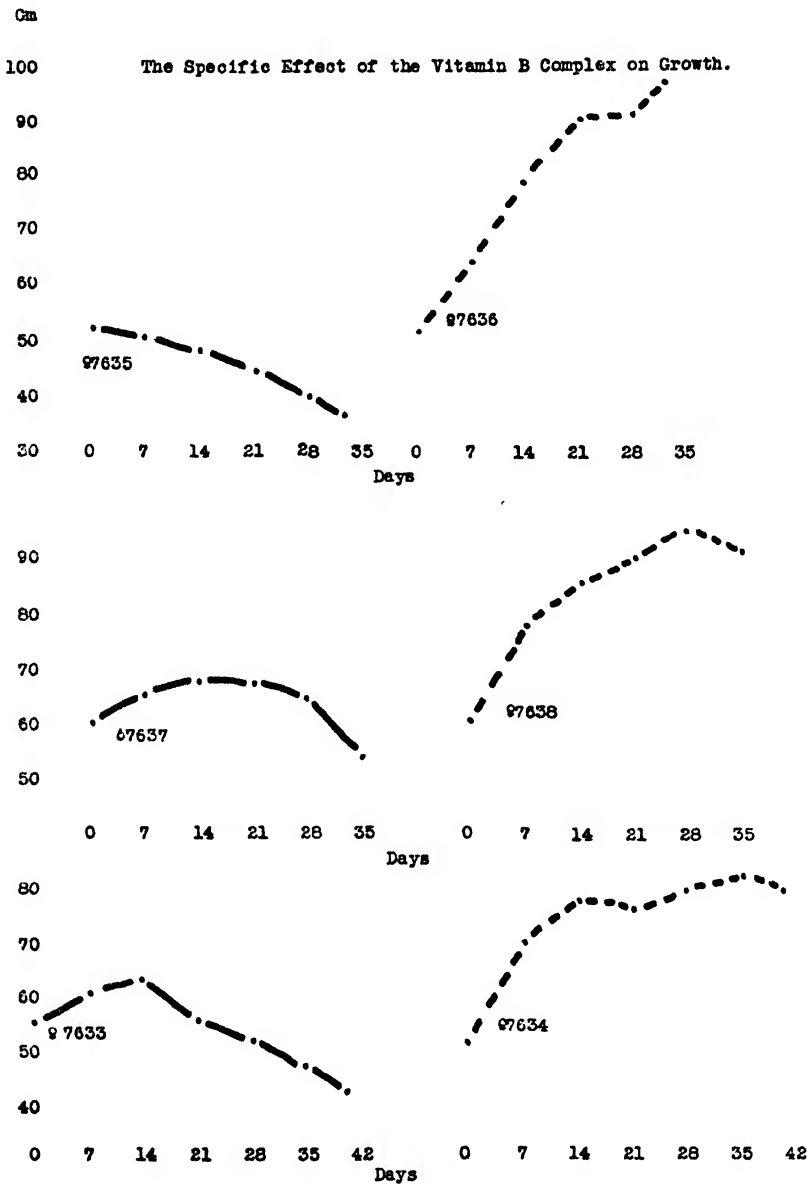


CHART 1. Curves in straight lines represent character of growth of animals on a ration satisfactory in every respect but deficient in the vitamin B complex. Curves in dash lines represent growth records of litter mate rats on the same diet fortified with 10 per cent dried Northwestern yeast as a source of vitamins B and G, and restricted to the same daily food and water intake as their litter mates on the ration deficient in the vitamin B complex.

Since ♀ 7480 (Chart 3) received the same daily amounts of food and water as its litter mate, ♂ 7479 (on the vitamin B-deficient ration) but in addition vitamin B, the growth made by ♀ 7480 must be credited spe-

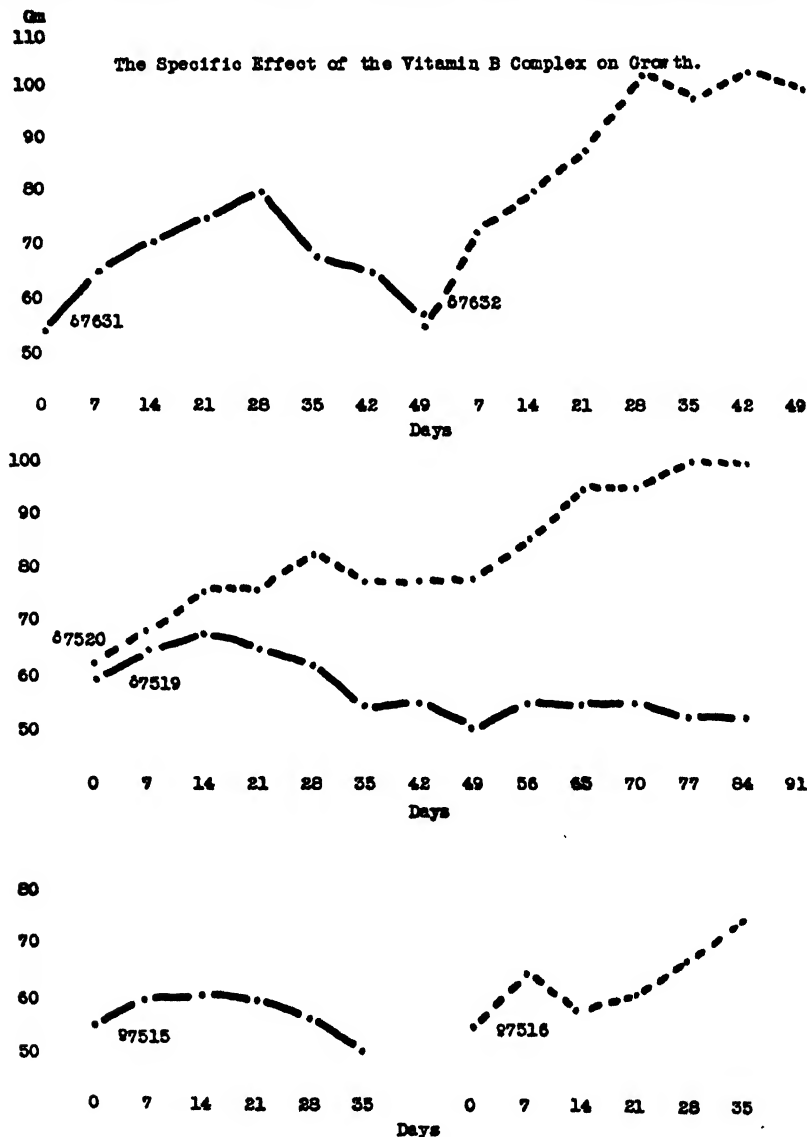


CHART 2. Curves in straight lines represent character of growth of animals on a ration satisfactory in every respect but deficient in the vitamin B complex. Curves in dash lines represent growth records of litter mate rats on the same diet fortified with 10 per cent dried Northwestern yeast as a source of vitamins B and G, and restricted to the same daily food and water intake as their litter mates on the ration deficient in the vitamin B complex.

cifically to this vitamin. The additional increment of growth made by litter mate, ♀ 7482, must be attributed to the fact that its diet was unrestricted, and the difference in the gain of weight between females 7480

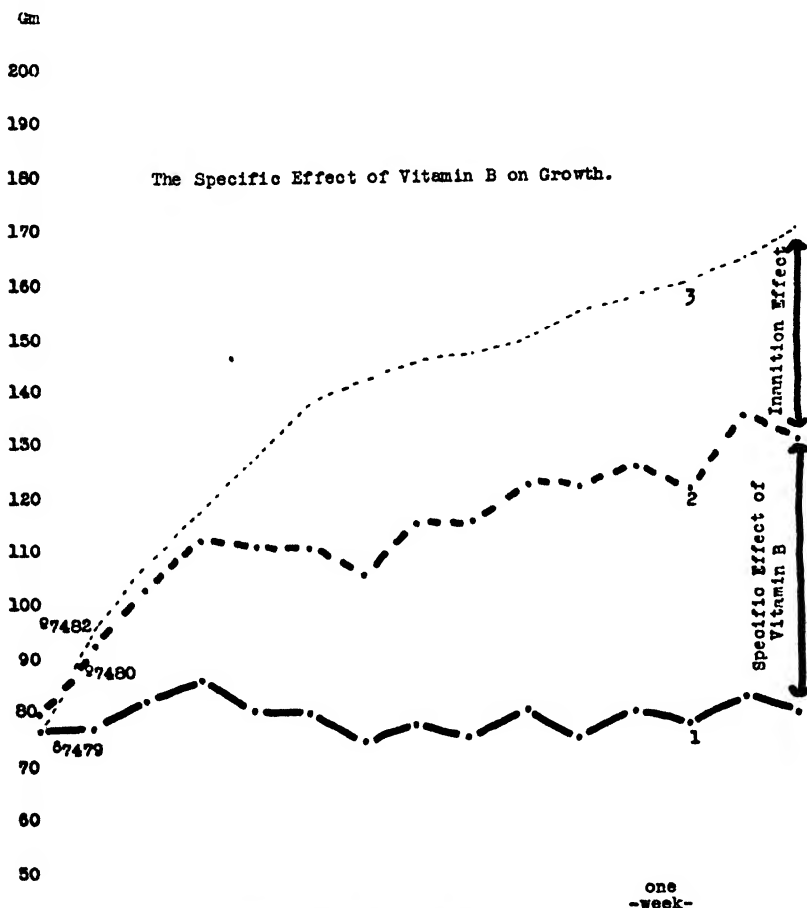


CHART 3. Curve 1 represents the character of growth of ♂ 7479 on a diet deficient only in vitamin B, 10 per cent Northwestern yeast autoclaved for 6 hours at 20 pounds pressure serving as a source of vitamin G; Curve 2 indicates the growth of litter mate, ♀ 7480, on the same diet fortified with 10 per cent of this yeast which has been untreated, and restricted to the same daily food and water intake as ♀ 7479; Curve 3 shows the growth of litter mate, ♀ 7482, on the same diet as ♀ 7480 but unrestricted to its plane of nutrition. A differentiation between the specific effect of vitamin B and the influence of inanition on growth is quite apparent. Similar results are shown in Chart 4.

and 7482 is designated as the inanition effect, since it signifies the growth ♀ 7480 failed to make because it was on a lower plane of nutrition. Similarly, in Chart 4 the difference in gain of weight of females 7445 and 7446

has been produced by the specific influence of vitamin B and the difference in weight between females 7446 and 7482 has been produced by higher plane of nutrition. We must, therefore, now recognize that vitamin B exercises two physiological functions: 1.—it plays a role in metabolism to

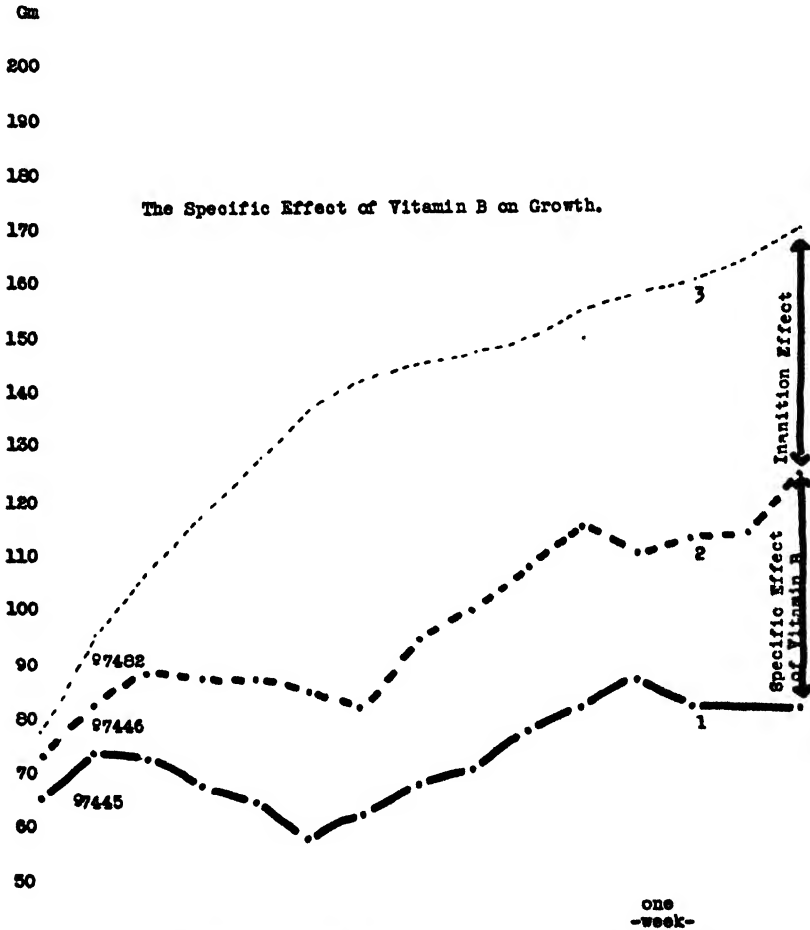


CHART 4. Curve 1 represents the character of growth of ♀ 7445 on a diet deficient only in vitamin B; Curve 2, the growth of litter mate, ♀ 7446, on the same diet fortified with an abundance of vitamin B and restricted to the same plane of nutrition as ♀ 7445; Curve 3, the growth record of ♀ 7482 on the same diet as ♀ 7446 but unrestricted to its daily food and water intake.

the extent that, just as some of the essential amino-acids, it is indispensable for growth without any reference to the food intake; and, 2.—it stimulates the appetite, so as to allow a sufficient intake of all the dietary factors essential for optimum function of all physiological processes in the body.

As little as 0.25 and 0.5 mg. daily of vitamin B concentrate, containing only 0.125 and 0.25 mg. of solids respectively, produced a specific effect on growth. (Chart 5)

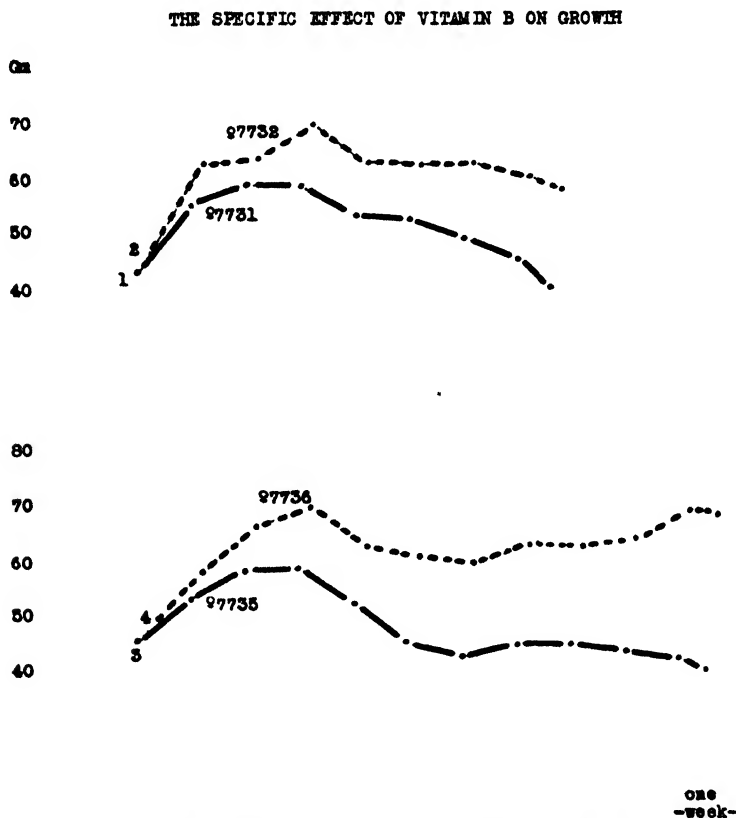


CHART 5. Curve 1 represents the character of growth of ♀ 7731 on a diet satisfactory in every respect but deficient in vitamin B, vitamin G having been supplied in the ration by 10 per cent Northwestern dried yeast autoclaved for 6 hours at 20 pounds pressure, during which process vitamin B was destroyed. Curve 2 shows the growth of litter mate, ♀ 7732, on the same diet and restricted to the same daily food and water intake as ♀ 7731 but ♀ 7732 in addition received daily separately from the ration 0.25 mg. of a highly concentrated vitamin B extract, containing 0.125 mg. solids.

Curve 3 represents the character of growth of ♀ 7735 on a diet deficient only in vitamin B. Curve 4, the growth record of litter mate, ♀ 7736, on the same diet and same plane of nutrition as ♀ 7735, but ♀ 7736 in addition received daily 0.5 mg. of a vitamin B concentrate containing 0.25 mg. solids.

Keeping the plane of nutrition constant, the *specific* effect of vitamin B on growth becomes quite apparent.

The application of our work to the human can be anticipated in borderline diseases associated with malnutrition, i.e., people with a fair appetite



may still be in need of vitamin B for optimum well being, since the exact physiological rôle of vitamin B in the organism is not yet clearly understood. At present we are finding the following pathological conditions as due to specific vitamin B deficiency: Marked reduction in the glycogen content of the liver, hemorrhages in the osteogenetic tissues and in the intestinal tract, and a lymphopenia with a corresponding polymorphonuclear leucocytosis (8).

#### SUMMARY

Vitamin B produces growth in two ways: 1.—It possesses the physiological function of stimulating growth *per se*, unrelated to food intake; and 2.—It produces growth by increasing the plane of nutrition through a stimulation of the appetite.

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# CHANGES IN THE WEIGHTS OF VARIOUS ORGANS AND SYSTEMS OF YOUNG RATS MAINTAINED ON A LOW-PROTEIN DIET

By

MARCIANO LIMSON AND C. M. JACKSON

*(From the Department of Anatomy, University of  
Minnesota, Minneapolis.)*

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IT IS known that when the normal growth of young animals is prevented by various types of inanition, peculiar dystrophic changes occur, with persistent growth in some parts and loss of weight in others (Jackson, 4, 5, 6). In some earlier experiments of Osborne and Mendel, no such changes (excepting possible growth of the nervous system) were found in young rats maintained for long periods on incomplete protein diets. Mendel and Judson (11), however, noted persistent skeletal growth in mice on inadequate protein diets. More recently Winters, Smith and Mendel (21) observed typical changes in the skeleton, and a few other organs, in young rats with growth retarded by diets deficient in protein, calories, or salts. The present study was undertaken to determine more comprehensively the effects of a prolonged low-protein diet upon the growth of nearly all of the various organs and systems. We are especially indebted to Professor George O. Burr for advice and assistance in regard to the diets used.

## MATERIAL AND METHODS

The rats used in this study were of pure Wistar albino stock, reared in the colony at the Institute of Anatomy of the University of Minnesota. A total of 48 rats from 9 litters was used. All were weaned and weighed on the twenty-first day of age, according to the usual routine. Of the 48 rats, 28 were used for the tests and 20 as normal controls, dividing each litter as evenly as possible for this purpose. In the 28 test rats, the average initial weight of the 14 males was 41.14 grams; of the 14 females 40.35 grams; total for both sexes 40.75 grams (range 35 to 48). In the 20 control rats, the average weight of the 10 males was 42.5 grams; of the 10 females 42.8 grams; of the total series 42.65 grams (range 35 to 50). The average weight of the controls was almost the same as the final weight of the test rats (42.42 grams), as shown in Table I.

The 20 control rats after weaning were killed immediately with chloroform. They were then autopsied, and their organs were carefully weighed.

TABLE I  
AVERAGE DATA FOR TEST AND CONTROL RATS AT AUTOPSY  
WEIGHT IN GRAMS, LENGTHS IN CENTIMETERS

Measurements	Control rats mean (10 m., 10 f.)	Test rats mean (14 m., 14 f.)	Percentage difference in test rats	Significance ratio: Difference
				P.E. diff.
Body-weight.....	42.65 $\pm$ 0.55	42.42 $\pm$ 0.41	- 0.5	0.3
Body-length.....	11.41 $\pm$ 0.07	12.53 $\pm$ 0.09	+ 9.8	9.8
Tail-length.....	9.10 $\pm$ 0.08	11.204 $\pm$ 0.095	+23.0	16.9

Organs showing significant increase

Spinal cord.....	0.2245 $\pm$ 0.0024	0.3575 $\pm$ 0.0041	+59.2	27.8
Eyeballs.....	0.1355 $\pm$ 0.0020	0.2085 $\pm$ 0.0044	+53.8	15.1
Stomach.....	0.2985 $\pm$ 0.0050	0.4331 $\pm$ 0.0080	+44.4	62.9
Skeleton (lig.).....	6.677 $\pm$ 0.035	9.170 $\pm$ 0.213	+37.3	20.6
Epididymides.....	0.0336 $\pm$ 0.00089	0.0452 $\pm$ 0.0019	+34.6	5.5
Hypophysis.....	0.00163 $\pm$ 0.000076	0.00214 $\pm$ 0.000046	+31.2	5.7
Liver.....	2.209 $\pm$ 0.050	2.569 $\pm$ 0.039	+16.3	5.6
Head.....	6.688 $\pm$ 0.089	7.066 $\pm$ 0.057	+ 5.6	3.6
Heart.....	0.2737 $\pm$ 0.0033	0.2864 $\pm$ 0.0026	+ 4.6	3.0

Organs showing insignificant changes

Kidneys.....	0.6881 $\pm$ 0.0110	0.7155 $\pm$ 0.0073	+ 3.9	2.1
Tongue.....	0.426 $\pm$ 0.047	0.435 $\pm$ 0.038	+ 1.9	0.2
Uterus.....	0.0327 $\pm$ 0.0015	0.0333 $\pm$ 0.00077	+ 1.8	0.4
Intestines.....	0.290 $\pm$ 0.035	1.306 $\pm$ 0.020	+ 1.2	0.4
Testes.....	0.2289 $\pm$ 0.0074	0.2312 $\pm$ 0.014	+ 1.0	0.1
Brain.....	1.494 $\pm$ 0.012	1.501 $\pm$ 0.0093	+ 0.5	0.5
Lungs.....	0.3850 $\pm$ 0.0082	0.3844 $\pm$ 0.0069	- 0.2	0.1
Suprarenals.....	0.0148 $\pm$ 0.00040	0.0137 $\pm$ 0.00017	- 7.2	2.4
Pancreas.....	0.3555 $\pm$ 0.0140	0.3185 $\pm$ 0.0092	-10.4	2.3

Organs showing significant decrease

Integument.....	5.925 $\pm$ 0.116	5.115 $\pm$ 0.072	-13.6	5.9
Prostate.....	0.0295 $\pm$ 0.0012	0.0254 $\pm$ 0.00035	-13.7	3.2
Musculature.....	12.955 $\pm$ 0.225	11.100 $\pm$ 0.308	-14.3	4.9
Ext. orbital glands....	0.0595 $\pm$ 0.0016	0.0439 $\pm$ 0.0019	-26.0	6.3
Submaxillary glands..	0.1794 $\pm$ 0.0034	0.1249 $\pm$ 0.0034	-30.3	11.2
Ovaries.....	0.0258 $\pm$ 0.0013	0.0136 $\pm$ 0.0017	-47.3	5.6
Spleen.....	0.1460 $\pm$ 0.0051	0.0755 $\pm$ 0.0016	-48.2	13.1
Thymus.....	0.1976 $\pm$ 0.0068	0.0703 $\pm$ 0.0025	-64.4	17.5

In general, the autopsy technic used by Jackson (7) was followed for both control and test animals. In the control rats, the body lengths, tail lengths, and weights of the various organs in most cases were in fairly close agreement with the norms of Donaldson (2) for rats of similar body weight.

The 28 test rats were placed in individual wire cages with wire net floors, allowing the feces to drop through. Body weights were taken daily at the same hour. City tap water was supplied *ad libitum* from bottles with curved tubular glass outlets. The special diet was started immediately after weaning. The basal diet was the following mixture:

	Grams
Sucrose.....	75.0
Salts (McCollum's mixture 185).....	4.5
Lard.....	20.0
Total.....	99.5

The basal diet was fed *ad libitum*. The consumption was measured by weighing the initial amount placed in the cage and later the residual amount left in the food cups together with that collected from a paper sheet placed beneath the floor of the cage. The daily average consumption of each rat for the week was divided by his corresponding average body weight to obtain the daily consumption per gram of body weight. Since no sex difference was found, the sexes were combined for the average consumption shown in Fig. 1.

The accessory diet included a mixture of brewer's yeast<sup>1</sup> and dried wheat germ<sup>2</sup> in equal parts, fed in amounts varying from 0.3 to 0.5 gram daily, as shown in Fig. 1. This mixture supplied the vitamin B complex, vitamin E, and also a limited amount of protein. The protein was supplemented, during the 11th week (period of 8 days only), by 0.3 gram of purified casein daily. For vitamins A and D, cod liver oil<sup>3</sup> was administered in dosage of 2 drops daily, increased to 4 drops during the 5th and 6th weeks. All the accessories were mixed and fed separately each day to insure the individual consumption of the amounts indicated.

The experiments extended from May to October, 1929. All of the 28 test rats were kept on the experimental diet at least 15 weeks; 21 of them were continued during the 16th week, and 10 were not killed and autopsied until the 17th week. In Fig. 1, the results are shown only to the 15th week, including all the animals.

In comparing the data for the test and control groups, the probable errors of the means were calculated, using the formulas of Pearl (16). The

<sup>1</sup> Northwestern pure dehydrated.

<sup>2</sup> Washburn-Crosby Co.

<sup>3</sup> Patch.

means were first calculated for the sexes separately, but as no significant difference appeared, the sexes were combined as shown in Table I. The percentage difference for the test rats in each case is found by subtracting the mean for the controls from the mean for the test rats, and expressing this difference (plus or minus) as a percentage of the control mean. In order to determine whether the differences are significant, in each case the absolute difference is divided by the probable error of the difference. The quotient is expressed as the "significance ratio" in the last column of

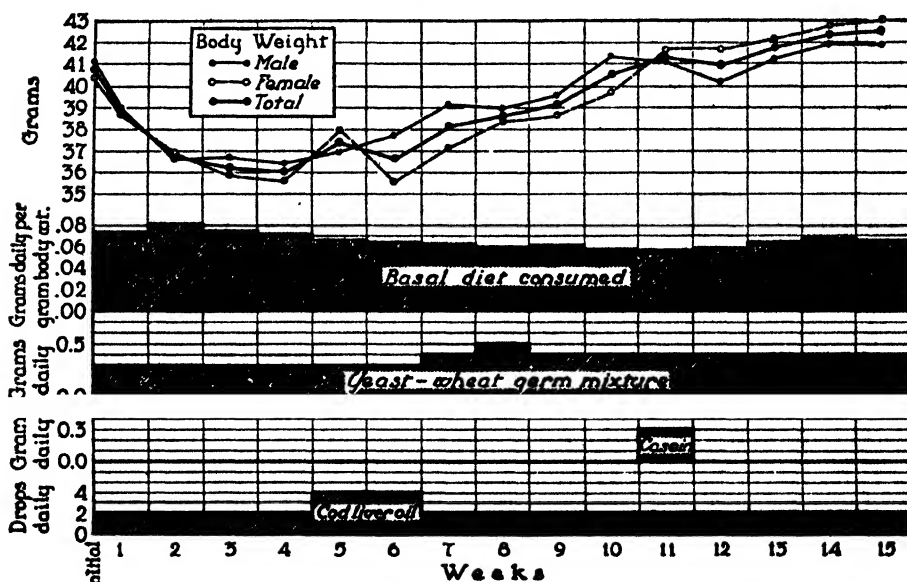


FIG. 1. In the upper part of the figure are curves showing the changes in average body weight for the test rats, male, female and total, for each week up to the fifteenth. The graphs below represent the corresponding intake of the basal diet (grams daily per gram of rat), of the yeast-wheat germ mixture (grams daily), of casein (grams daily, 11th week only), and of cod liver oil (drops daily).

Table I. If this ratio exceeds 3, there is less than one chance in 20 that the difference is due to accidental variation (assuming normal distribution), so the difference is held to be statistically significant.

#### GENERAL OBSERVATIONS

In general, all of the test rats continued in good health throughout the experimental period of about four months of repressed growth. The external appearance varied somewhat, but there was progressive tendency toward emaciation as the experiment continued. In some cases the rats finally became markedly emaciated, with the characteristic dorsal con-

vexity of the vertebral column. Throughout the experiment most of the rats appeared more than normally active, although there was in some cases a more quiet and drowsy appearance toward the end. The appetite usually remained fairly good, and the daily accessories (yeast, wheat germ and cod liver oil) were consumed greedily when placed in the cages. The hair coat and eyes remained normal. No evidences of vitamin deficiency (ophthalmia, rickets, polyneuritis, pellagra) were noted during the course of the experiment or at autopsy.

### BODY WEIGHT AND DIETARY RELATIONS

*Body weight.* The curves of body weight (Fig. 1) show no consistent sex difference, so that in the following discussion the average will be taken for the sexes combined. It is evident that the mean body weight declined steadily from 40.75 grams at the beginning to about 36 grams during the 3rd and 4th weeks. This corresponds to an average loss of nearly 12 per cent, the individual losses ranging from 8 to 25 per cent of the initial weight. Thereafter there was a slow but fairly steady gain in average weight. In the 11th week it slightly surpassed the initial weight, averaging 41.3 grams. It decreased slightly to 40.9 grams in the 12th week, but increased thereafter to 41.7 grams in the 13th week, 42.3 grams in the 14th and 42.4 grams in the 15th week. The final average for the test rats (some continued into the 16th and 17th weeks) was 42.42 grams, as given in Table I. The final individual weights ranged from 35 to 46 grams in the test rats, and from 35 to 50 grams in the normal controls.

*Dietary relations.* In relation to the changes in body weight of the test rats, the adequacy of the total food intake (caloric value) as well as of the fat, salts, protein and vitamins should be considered. The water intake was not measured, but may be assumed to be adequate, as it was supplied *ad libitum*. Unfortunately the same assumption cannot be made for the total consumption of the basal diet similarly supplied *ad libitum*, since it is known that the voluntary food intake may decrease when necessary factors are lacking.

In the present experiments, as shown in Fig. 1, the voluntary daily consumption of the basal diet averaged 73 milligrams per gram of rat during the first week, increasing to 82 milligrams during the 2nd week. Thereafter, it slowly decreased to a minimum of 57 milligrams in the 11th week, rising again to about 66 milligrams in the 14th and 15th weeks. If we add the weight of the yeast-wheat germ mixture and of the casein (11th week only), the daily total food intake (aside from the cod liver oil) per gram rat becomes 81 milligrams in the first week, 90 in the 2nd,

82 in the 3rd, decreasing to 68 milligrams in the 10th week. With the casein in the 11th week the daily intake per gram rat rises to 74 milligrams, followed by 68, 74, 77, and 75 milligrams in the 12th to the 15th weeks, respectively.

The average daily intake of basal diet in grams per rat for the 15 consecutive weeks (in order) was 2.85, 3.01, 2.68, 2.56, 2.52, 2.35, 2.34, 2.33, 2.37, 2.34, 2.34, 2.39, 2.68, 2.83, 2.78.

It seems unlikely that the decline in body weight during the first three weeks was due to inadequate energy intake, as the consumption per gram of rat at this time (even without the cod liver oil) was equal to or greater than that found by Osborne and Mendel (12) to be adequate for maintenance in rats of similar size (30 to 50 grams) on diets of similar caloric value. Wang, Huddlestun, and Saphir (20) obtained some growth for 20 weeks with a food intake of only 58 milligrams per gram rat per day, but the exact composition of this food is not stated. The adequacy of the energy intake in our tests would appear to be evidenced also by the slight but continued growth later upon a slightly decreased relative food intake. But in this connection, as observed by Jackson (4) and others, it must be remembered that the food intake required for maintenance in growing animals decreases notably as the experiment continues, probably on account of lowered basal metabolism.

The necessary fat (as shown by Burr's work) was provided by the lard in the basal diet. For the adequacy of the salt intake we have no definite evidence, beyond the fact that the proportion used in the basal diet has been found amply sufficient for growing animals with larger intake of adequate diets. The actual amount of the salt mixture required for maintenance is not definitely known, but Osborne and Mendel (13) and Winters, Smith, and Mendel (21) have shown that growth in rats can be suppressed or retarded by low salt diets.

The protein content of the diet, excepting the 11th week, was supplied solely by the yeast-wheat germ mixture. According to the analyses cited by Osborne and Mendel (14) and Horwath (3), the actual protein content will scarcely exceed one-third in the case of either yeast or wheat germ. According to this estimate the protein content for 0.3 gram of the mixture would be 100 milligrams. On this basis, the protein intake per gram rat per day during the 15 weeks (in order) averaged as follows in milligrams: 2.6, 2.7, 2.8, 2.8, 2.7, 2.7, 3.5, 4.3, 3.4, 3.3, 10.5, 3.3, 3.2, 3.2, 3.2. Excepting the 11th week (when the casein was added), this is far below the minimum casein intake (about 12 milligrams daily per gram rat) recorded by Osborne and Mendel (12) in rats maintained at similar weight (30 to 50

grams). Wang, Huddlestun, and Saphir (20) found 4 milligrams of casein daily per gram rat to be sufficient protein for slow growth in rats of 50 to 60 grams, but the casein was apparently "supplemented by a definite amount of yeast and lettuce," the protein content of which is not stated. With only 2 milligrams of casein, their rats lost weight. Although our rats lost weight during the first two or three weeks, the protein intake was sufficient thereafter to permit maintenance and even a slight increase in weight, as is apparent in Fig. 1.

The increase in body weight of our rats on such low protein intake is somewhat difficult to explain, unless we assume that the proteins of the yeast-wheat germ mixture are superior to casein. Osborne and Mendel (14) found the proteins of the wheat germ more efficient than those of the entire kernel in promoting growth of rats. Osborne and Mendel (15) and others have kept growing rats over long periods on diets in which yeast furnished the sole source of nitrogen as well as of water-soluble vitamin. This shows that yeast protein (in sufficient quantity) is adequate for growth. Still and Koch (19), however, observed that the growth of young rats on diets containing yeast as the sole source of the protein is less satisfactory than that when the yeast is replaced by casein. Nevertheless they found no special amino acid deficiency in the yeast protein. If protein was the limiting factor retarding growth in our rats, it is surprising that the addition of casein had so little effect in the 11th week, but possibly the time allowed was insufficient.

Finally the adequacy of the vitamin content of the diet must be considered. Two drops of cod liver oil<sup>4</sup> daily was assumed to supply vitamins A and D in amounts adequate for slight growth at this level, in accordance with the experience of Burr.<sup>5</sup> Moreover, slight growth on this dosage did occur in the latter half of the experiment. The slight, apparent response when the oil was increased to four drops in the 5th week is therefore probably insignificant, especially as the body weight decreased slightly in the following week on the same dosage (Fig. 1). Similarly Burr has found the 0.3 gram yeast-wheat germ mixture daily ample to supply the vitamin B complex and vitamin E for moderate growth. It is therefore improbable that the slight improvement in growth from the 7th week onward was due to the increase in these factors with the increased amount of the yeast and wheat germ. On the whole, it appears more probable that the limiting factor was rather in the protein content. However, it must be frankly ad-

<sup>4</sup> Patch.

<sup>5</sup> Personal communication.



mitted that other deficiencies may have existed. Further work would be necessary to settle this question definitely.

#### ORGAN WEIGHTS

In Table I are given the mean organ weights for the test rats and controls, the percentage change in the test rats, and the significance of this change as indicated by the significance ratio. For convenience, the organs of the test rats are grouped under those showing 1.— a significant increase; 2.— insignificant change; and 3.— a significant decrease.

*Organs showing significant increase.* These include, in decreasing order, the spinal cord, eyeballs, stomach, ligamentous skeleton, epididymides, hypophysis, liver, head and heart. The increases in weight (estimated by comparison with the weights in the normal controls of the same body weight) range from 59.2 per cent in the spinal cord down to 4.6 per cent in the heart. The marked increase of 37.3 per cent in skeletal weight is in accordance with the increase of 9.8 per cent in body-length, measured from nose to anus. That this persistent skeletal growth varies in different regions of the body is indicated by the greater relative increase of 23 per cent in the tail-length.

*Organs showing insignificant changes.* As indicated in Table I, the organs showing but slight changes in weight are the kidneys, tongue, uterus, intestines, testes, brain, lungs, suprarenals, and pancreas. The increase of 3.9 per cent in the kidneys, and the losses of 7.2 and 10.4 per cent, respectively, in suprarenals and pancreas, might be considered as probably (but not certainly) significant, since the significance ratio in these cases is between two and three.

*Organs showing significant decrease.* The organs in this group, in order of increasing relative losses in weight, are the integument, prostate, musculature, external orbital glands, submaxillary glands, ovaries, spleen and thymus. The losses range from 13.6 per cent in the integument to 64.4 per cent in the thymus.

#### DISCUSSION

The results of the present study may be compared with those of previous investigations, including those in which growth of rats was repressed or retarded by dietary deficiencies of protein, calories, water, salts, or vitamin D (rickets).

The effects of protein deficiency on the weights of a few organs were observed by Winters, Smith, and Mendel (21). In young rats maintained several weeks on an inadequate protein diet, they found an average increase of about 4 per cent in body length, with a corresponding increase of

50 to 60 per cent in the weight of the skeleton (leg bones). Increases (usually slight) were noted also in the brain, liver, heart and lungs, and especially in the kidneys (55 per cent). Wang, Huddlestun, and Saphir (20) found that on very low protein diets, rats at 50 to 60 grams body weight became markedly emaciated and inactive, mostly dying at the 16th to 22nd week. At somewhat higher protein level, the body weight doubled in this time. At all levels of protein intake the kidneys remained normal in weight, in agreement with our results for low protein diet but in contrast with those of Winters, Smith, and Mendel.

Jackson (4) held rats at about 24 grams in body weight by general under-feeding from 3 to 10 weeks of age. The average changes in body length and organ weights were remarkably similar to those found in the present study. Marked increases occurred in the eyeballs, spinal cord, testes, skeleton stomach-intestines, suprarenal glands, hypophysis, and liver. Slight changes (below 10 per cent) were noted in the head, kidneys, musculature, brain, and heart. Decreases were found in the lungs, ovaries, skin, spleen, and thymus. As compared with the present results on low-protein diet, the main differences were in the testes and suprarenals, showing marked increases of 34 and 26 per cent, respectively; and in the skin, with a loss of 36 per cent. Winters, Smith, and Mendel (21) in young rats maintained on low-calorie diet similarly found skeletal growth, with increase of 4 per cent in body length and of 50 to 60 per cent in weight of leg bones. The brain showed slight increase, but the kidneys increased 55 per cent. The other organs were not mentioned. These results coincide with those found by them in low-protein tests.

Kudo (10) similarly maintained rats averaging 27 grams over a period of 13 weeks by low-water diet. The changes in organ weights were in general similar to those found in the present study, but there are some exceptions. The testes decreased 49 per cent in weight, the musculature only 4 per cent. On the other hand, the kidneys increased 58 per cent and the suprarenals 68 per cent. Jackson and Smith (9) more recently retarded rats of 50 to 70 grams by deficient water intake for longer periods of 16 to 33 weeks. The changes in organ weights resembled those found by Kudo, but the testes increased 70 per cent while the liver lost 30 per cent.

Comparison may also be made with the results of Jackson and Carleton (8) in rats 4 to 8 weeks old, body weight 30 to 75 grams, stunted in growth by a rachitic diet. The skeleton, in spite of the rachitic changes, averaged 15 per cent above normal weight. The eyeballs, suprarenals, kidneys, lungs, heart, ovaries, and submaxillary glands were also above normal. The head, testes, stomach-intestines, liver, spleen, and lungs were nearly

normal, while the hypophysis, musculature, skin, and thymus were definitely subnormal in weight. The changes in organ weights are in part similar to those found in protein deficiency, but there are several obvious differences.

In rats retarded by low-salt diets, Winters, Smith, and Mendel (21) observed an increase of 3 per cent in average body length, in spite of a loss of 30 to 40 per cent in skeletal weight (leg bones). A slight increase in brain weight and a marked gain of 55 per cent in the kidneys duplicated their findings with inadequate protein and low calorie diets. In similar tests with low-salt diets, Smith and Schultz (18) likewise noted increase in body and tail lengths, and in skeletal weight, with striking enlargement of the kidneys. The spleen decreased in weight.

In general, the foregoing results indicate clearly that during repression of growth in body weight by various types of dietary deficiency, there are striking dystrophic changes among the individual organs, some increasing markedly, while others undergo atrophy (Jackson, 5, 6) (Smith, 17). When the results in various dietary deficiencies are compared, it is evident that the changes in organ weights in many cases are remarkably similar. The similarities in organ changes under varied types of inanition may be due to a common underlying factor—interference with the general process of nutrition. This interference is generally associated with a reduced food intake, which may overshadow the more specific effects of deficiencies in the individual nutritional elements concerned.

The differences in organ changes recorded under various nutritional deficiencies may be in part ascribed to accidental variations, or to differences in the age or strain of the animals, length of the experiment, etc. In part, however, the differences in organ response are probably due to more specific failure to supply the nutritional requirements of these organs. Such nutritional requirements differ considerably in different organs, and even in the same organ at different ages and stages of development. Nutritional deficiencies can therefore upset the normal growth process by permitting some organs to continue growth while others remain stationary or undergo atrophic changes.

#### SUMMARY

1. Twenty-eight rats were fed from weaning time (age 3 weeks) a diet low in protein but planned to be adequate in calories and in the various accessories. The rats remained healthy and active over a period of about 4 months. No sex difference or evidence of specific nutritional disorder was apparent. The average body weight decreased from about 41 grams to 36

grams by the end of the first month, thereafter increasing slowly to about 42 grams at the end of the test.

2. Finally the test rats were killed and autopsied, and likewise 20 normal control rats of the same body weight for comparison. The average body length of the test rats had increased about 10 per cent, and the tail length 23 per cent, in comparison with the controls.

3. The average organ weights are considered in 3 groups. Those showing a significant increase in weight in the test group are (in order from greatest to least change): spinal cord, eyeballs, stomach, skeleton, epididymides, hypophysis, liver, head, and heart. The changes vary from about 59 per cent in the spinal cord to 5 per cent in the heart.

4. The organs showing but slight (statistically insignificant) change are the kidneys, tongue, uterus, intestines, testes, brain, lungs, suprarenals, and pancreas.

5. The organs showing significant decrease in weight are (in order) the integument, prostate, musculature, external orbital, and submaxillary glands, ovaries, spleen, and thymus. The losses vary from about 14 per cent in the integument to 64 per cent in the thymus.

6. The dystrophic changes in organ weights in many respects show a remarkable degree of correspondence in various types of nutritional deficiency. This may be due to the common underlying factor of interference with the general process of nutrition, usually associated with a deficient intake of food. On the other hand, differences in the changes among the various organs (comparing different organs during the same nutritional deficiency, or the same organ during different deficiencies) may be due to specific differences in their nutritional requirements.

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# FAILURES TO PRODUCE EXPERIMENTAL DENTAL CARIES IN THE WHITE RAT WITH HIGH CARBOHYDRATE DIET AND BACILLUS ACIDOPHILUS OR WITH VITAMIN D DEFICIENCY

BY C. A. LILLY

*(From the Department of Internal Medicine, University of Michigan, Ann Arbor.)*

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**N**O PROBLEM is of greater interest to humanity today than is that of dental caries. And no problem is older. Three thousand years B.C. the Egyptians were using a preparation of flint, leaves, and honey to prevent caries. At the same time the Chinese were using musk and salt for the same purpose. Aristotle thought sweet, soft figs decayed the teeth. Hippocrates held "humors of the body" responsible for caries. Pliny recommended cleaning the teeth to prevent their decay. The Romans advocated the use of tooth picks and dentifrices, as did the Arabians. In the middle ages caries was attributed to a "worm in the tooth." In 1728 horse hair tooth brushes were condemned as harmful by the French. Thomas Berdmon, dentist to King George III, advocated soft tooth powder to clean the teeth and for the prevention of caries. Westcott, in 1843, showed that acids originated from fermentation and thought that such acids dissolved the enamel and produced caries. Harris, in 1853, and Tomes, in 1873, ascribed caries to acids produced by fermentations.

Miller (1) advanced his important parasite theory, and claimed that organisms in the mouth act on carbohydrate materials and by a process of fermentation form lactic, butyric, acetic, and other acids which destroy the enamel. McIntosh, James, and Lazarus-Barlow (2) postulated a constantly present bacillus capable of producing acids by fermenting carbohydrate material, and reported that this bacillus resembled the acidophilus group of Moro. Bunting (3) reported a series of studies in which he believed he had correlated the formation of dental caries with the activities of the *Bacillus acidophilus*. Later, he (4) published further studies to the effect that certain individuals appear to have an immunity to the *Bacillus acidophilus*, but that this immunity may disappear over varying periods of time, after which caries may ensue. McCollum (5) several years ago called attention to the importance of scientific nutrition, during the period of growth, as well as to the proper nutrition of the expectant and nursing mother in order to develop sound teeth in the offspring.

Other observers have attributed the etiology of dental caries to a deficiency of vitamin C. The outstanding exponents of vitamin C deficiency in relation to caries are Zilva and Wells (6), who reported changes in the teeth of guinea pigs that had been fed a scorbutic diet. Höjer (7) produced dental lesions in guinea pigs by the use of vitamin C-deficient diets. P. R. Howe (8) reports the production of dental caries in monkeys fed a scorbutic diet. Hanke (9) accepts the findings of Howe and Höjer.

May Mellanby (10, 11, 12) has extensively studied the relationship of vitamin D to the calcification of teeth in puppies, and concludes that a deficiency of vitamin D results in the production of imperfect (hypoplastic) teeth, which are subject to early decay.

It has now been pointed out that some workers attribute caries to the action of an organism; others believe it to be due to a deficiency of vitamin D; and still others to a deficiency of vitamin C. The first two hypotheses have not received general acceptance. Thus, McIntosh, James, and Lazarus-Barlow (13) failed to produce caries in rabbits and monkeys with *Bacillus acidophilus*. Rosebury and Karshan (14) studied the relationship of diet to dental caries. Their experiments were conducted over periods of forty to sixty days, and in these short periods they were unable to produce caries. Their results were as follows:

Sixty young rats were studied in three groups, in which were tested, respectively, a low-calcium, vitamin D-free diet, and a diet high in fermentable carbohydrate (all synthetic), in each case with and without the addition of human oral *Lactobacilli* fed in an adhesive paste. All rat mouths were cultured at intervals for aciduric organisms. The character of the growth obtained with the different diets is recorded. The presence of rickets in the first group was ascertained by line tests and ash determinations on the tibias. Aciduric bacteria similar to *Lactobacillus acidophilus* were found to be a normal inhabitant of the rat mouth, their presence being constant and independent of the feeding of human *Lactobacilli*. No dental caries was produced, in spite of evidence of retention of food in molar pits, probably indicating the absence in these experiments of one or more factors in susceptibility to caries.

We regarded the white rat as a poor animal in which to study vitamin C deficiency on account of its apparent ability to survive normally on a diet deficient in this vitamin. We therefore elected to study the other two possible postnatal factors, namely, a high carbohydrate diet and *Bacillus acidophilus*, and a vitamin D deficiency.

Because of the lack of agreement among investigators regarding the relationships of the various factors to caries, it seemed important to re-examine the question with the full knowledge that much of the confusion is attributable to the brevity of the feeding experiments. The decisive work of the past few years in the study of chronic diseases has emphasized the point that success depends at least in part on prolonged trials of any dietary factors.

Since some observers have attributed dental caries of offspring to the faulty nutritional condition of the mother during gestation and nursing, while others emphasize dietary abnormalities in effect after weaning, it was desirable to separate the undertaking into two divisions. For the present we chose to study, over long periods of time, the problem in the white rat after weaning. In order to avoid harmful conditions before this age had been reached, the experimental animals were selected according to the following plan: All animals used were at least the fifth generation of an inbred stock that had been fed a diet, *ad libitum*, consisting of fresh meat, whole milk, bones, bread, and fresh and cooked vegetables. The success of this diet is evidenced by the large size of the young at weaning, since when thirty days old the rats weighed from 55 to 70 grams each, depending on sex. The animals were kept in a large, well ventilated and well lighted room in wire cages, which were cleaned daily. With these animals, thus produced, two major questions were studied.

#### SERIES I

The animals of this series were placed on the experimental diet when thirty days old. Six groups of animals were employed. Group 1 (12 rats) received a diet originally devised by Mendel and used subsequently with satisfaction by many other observers as a normal diet for the growth and maintenance of the white rat. It consisted of:

	per cent
Starch	53
Lard..	25
Casein	18
Mendel's normal salt mixture.. .	4

With this, 0.03 per cent by weight of Viosterol (potency 100 D) was incorporated. In addition each rat received daily 10 grams of fresh green cabbage, 1 gram dried brewer's yeast, and tap water *ad libitum*.

Group 2, (12 rats). These animals received the same diet as group 1, with the exception that the starch of the Mendel diet was replaced by sucrose. Both groups were infected *per orum* with a pure culture of *Bacillus acidophilus* at the beginning of the test. The rats were kept on this diet for nine months, at which time they were killed and examined for dental caries. No caries was found.

In the next four groups, cabbage was replaced by lettuce. Otherwise groups 3 and 4 received the same diet as group 1, while groups 5 and 6 received the same diet as group 2.



In addition each rat in group 3 (starch) and in group 5 (sucrose) had about 1 cc. of a pure culture of *Bacillus acidophilus* forced into the mouth and rubbed over the teeth and gums with a large wire loop three times a week throughout the entire period of the feeding. There were 7 rats in each of these four groups. These 4 groups were fed the diet for one year (or until the animals were 13 months old), and were then killed and examined for



FIG. 1. A typical section of a mandibular molar of a rat that received a high carbohydrate diet and tri-weekly inoculations of *Bacillus acidophilus*. The dental tissue is entirely normal. On gross inspection a possible lesion was suspected, but the microscopic examination showed that the suspicious area was merely foreign material in an occlusal pit.

caries. After a careful search of all the teeth of all the rats of these groups, no single example of caries was found.

## SERIES II

Steenbock's rachitogenic diet No. 2965 was fed to ten rats that were kept in individual wire cages in a dark room. Four of these rats received 0.03 per cent Viosterol (potency 100 D), which was thoroughly incorporated in the diet. One rat died in 6½ months, the remaining three were killed after one year of feeding, or when they were thirteen months old. No

x-ray evidence of rickets, bone or joint deformities was discovered in this group.

The remaining six rats, that received Steenbock's rachitogenic diet only, exhibited the usual appearances produced by a rachitogenic diet. One rat lived 3 months, four others lived 4,  $5\frac{1}{2}$ ,  $8\frac{1}{2}$  and 11 months respectively and the sixth rat was killed after one year of feeding, or when



FIG. 2. A similar preparation from a rat that received a rachitogenic diet in the absence of vitamin D for one year. The rat was kept in a dark room throughout the feeding period. The tooth is entirely normal.

thirteen months old. All six of these animals showed x-ray evidence of florid rickets, with marked bone and joint deformities. The teeth in all the rats of this series were examined for caries and none found.

#### *Preparation of the teeth*

The superior and inferior maxillae were removed, the soft tissues dissected away and cleaned with water, a tooth brush and tooth powder. The teeth under illumination and magnification were then scrutinized for defects. All teeth showing the slightest suggestion of carious lesions were ground longitudinally by means of a dental engine and a fine carborundum

wheel to a plane bisecting the center of what appeared to be the carious lesion. This ground surface was then polished smooth by means of a crocus disc. The teeth were then imbedded on a glass slide by means of water-soluble glass, the polished surface toward the slide. The reverse surface was then ground down by means of a fine sandpaper disc until an approximate thickness of fifty micra was attained. This surface was then polished with a crocus disc. The section was then removed with water and placed in alcohol and then mounted with balsam without staining. Study of the sections was then made microscopically. What had appeared, microscopically, to be carious areas, proved on examination to be accumulations of an amorphous debris in the occlusal pits. No caries was found.

I am glad to take this occasion to thank Dr. L. H. Newburgh for the interest and advice given me throughout this investigation.

#### CONCLUSION

1. White rats, fed for one year on a normal diet (Mendel's), developed no dental caries.
2. White rats, fed for one year on a diet 53 per cent of which was sugar (sucrose), but otherwise normal, developed no dental caries.
3. White rats fed for one year on a diet 53 per cent of which was sugar, but otherwise normal, and which received, triweekly, oral inoculations of a pure culture of *Bacillus acidophilus*, developed no dental caries.
4. White rats which survived on a rachitogenic diet for long periods (8 months to one year) developed florid rickets with extreme bone and joint deformities, but no dental caries.

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# THE ANALYSIS OF THE CURVE OF HEAT PRODUCTION IN RELATION TO THE PLANE OF NUTRITION

By

E. B. FORBES AND MAX KRISS

(*From the Institute of Animal Nutrition, Pennsylvania State College, State College, Pa.*)

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## INTRODUCTION

**I**N RECENT studies at the Pennsylvania Institute of Animal Nutrition (1,2) the energy metabolism of cattle was studied at seven planes of nutrition, namely, 1.—fasting, 2.—half of the maintenance requirement, 3.—maintenance, 4.—half more than maintenance, 5.—two times maintenance, 6.—two and a half times maintenance, and 7.—three times maintenance; and was described in the second of the papers cited in language as follows (2, p. 77):

With the heat production of the fourth day of inanition as the base value, the heat production increased slowly between fasting and maintenance, and much more rapidly above maintenance, but with a decreased rate of rise between the planes of twice and three times maintenance.

The curve of heat production in relation to the plane of nutrition was found, therefore, to be a reversed or S curve.

The heat production of fasting being considered as including two factors, a waste heat of utilization of body nutrients katabolized, and a theoretical minimum base value, including no such waste—the curvature of the line of heat production in relation to increasing food consumption is interpreted as resulting from: 1.—the increasing concentration of metabolites circulating in the blood; 2.—the change in the proportions of protein, fat, and carbohydrate katabolized, with increase in the katabolism of food nutrients and decrease in the katabolism of body nutrients; 3.—the energy expense of synthesis of body nutrients (fat from carbohydrates); and 4.—the decreased metabolizability of the food at the higher planes of nutrition.

The idea of a hypothetical minimum heat production, less than the heat production of fasting, is suggested by the character of the curve of heat production as viewed from its upper end. From this point of view, as the katabolism of body substance begins to contribute a part of the heat, the direction of curvature is reversed and the degree of curvature is prominently and progressively increased—thus revealing the influence of a non-rectilinear component—the specific dynamic action of body substance katabolized.

These curves of heat production are presented in Figs. 1 and 2, and the data on which they are based are given in the first and the last columns of Table I.

In the papers referred to it was recognized that on account of the progressive replacement of food nutrients by body nutrients between main-

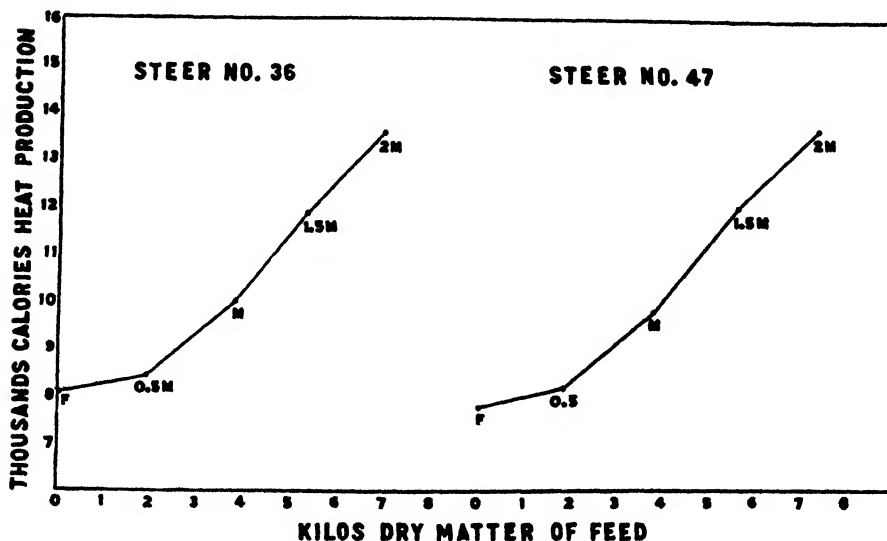


FIG. 1. The curve of heat production of cattle in relation to the quantity of feed eaten.

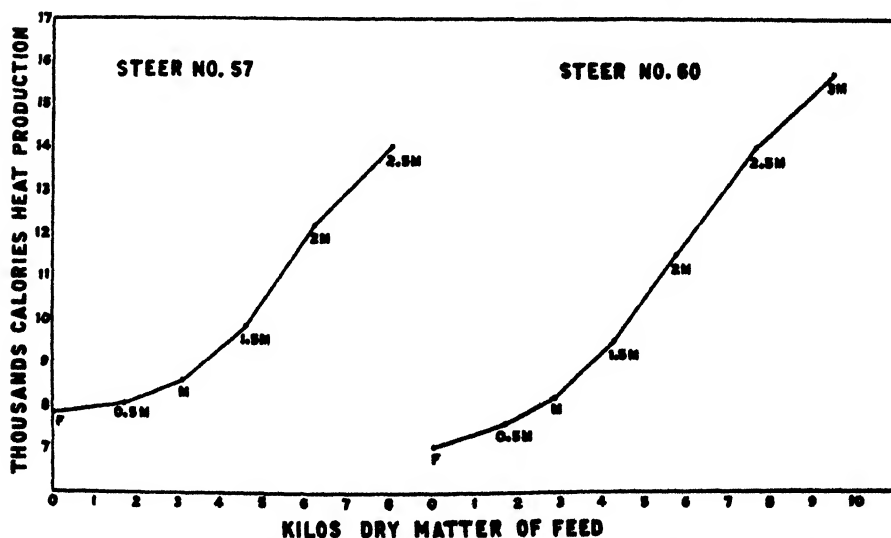


FIG. 2. The curve of heat production of cattle in relation to the quantity of feed eaten.

tenance and fasting, the heat increments at the lower planes of nutrition, as related to the food, were less than the true energy expense of food utilization by the amount of the decrement of heat of dynamic action of body

nutrients katabolized between the same planes of nutrition; and that the curvature of the line representing the relation of the heat production to the food consumption between fasting and maintenance is determined by the excess of the waste heat of utilization of increasing food nutrients as compared with that of decreasing body nutrients katabolized.

The break in the curve at the maintenance point may be due to a greater specific dynamic effect of food carbohydrates than of body fat, in cattle; and the reversed curvature at the higher points of observation appears to be due mainly to diminished metabolizability of the ration.

It is true that it is illogical, in a sense, to relate the heat production to a source (food nutrients) which produces all of it at maintenance and above, and none of it at fasting; but the practical interest in the food as the usual source of heat justifies this procedure. The more logical reference of the heat production to the energy of the nutrients katabolized, regardless of source, yields a curve which differs from the one discussed above only between fasting and maintenance as a result of the shortening of the abscissas—having the effect to increase the rate of rise in the curve between these two points, though this rate of rise remains less than that which prevails above maintenance.

The best support of the validity of these curves derives from the fact that whereas in experiment No. 238 (steers No. 36 and 47) the sequence of the experimental periods, as indicated by the plane of nutrition, was 2.0 maintenance, 1.5 maintenance, 0.5 maintenance, 1.0 maintenance, and fasting, in experiment No. 240 (steers No. 57 and 60) the sequence was radically changed, to the following order—1.0 maintenance, 1.5 maintenance, 2.0 maintenance, 2.5 maintenance, 3.0 maintenance, 0.5 maintenance, and fasting.

In order to make certain that the measurements of the fasting heat production in the two experiments might not possibly be affected by previous feeding at different planes of nutrition, the fasting periods in experiment No. 240 were preceded by preliminary feeding at a plane of maintenance, during which no heat measurements were made.

It is inconceivable that the curves representing the two experiments could have been so closely similar, under the circumstances, if the heat production, as determined, had not been characterized by a very nearly uniformly high degree of validity as representing the quantities of food consumed.

A research conducted by Gigon (3), contributes to the understanding of the relation of heat production to plane of nutrition. In a series of experiments he measured the energy metabolism of a man when fed casein ex-



TABLE I  
PARTITION OF THE ENERGY OF THE AVERAGE DAILY FEED AND THE HEAT PRODUCTION OF CATTLE AT DIFFERENT PLANES OF NUTRITION

Experiment, animal and period No.	Plane of nutrition	Dry matter of feed	Gross energy	Energy of feces	Energy of urine	Energy of methane	Digestible energy	Metabolizable energy	Heat increments	Heat production*
		Kgm.	Cals.	Cals.	Cals.	Cals.	Cals.	Cals.	Cals.	Cals.
Exp. 238 Steer 36	Period 12									
	" 6	—	—	—	—	—	—	—	—	8,060
	" 8	1.885	8,517	2,327	463	831	6,190	4,896	374	8,434
	" 4	3.762	16,997	4,466	802	1,481	12,531	10,248	1904	9,964
	" 2	5.353	24,189	6,669	1,043	1,842	17,520	14,636	3787	11,847
Steer 47	Period 11									
	" 5	—	—	—	—	—	—	—	—	7,790
	" 7	1.863	8,417	2,262	432	827	6,155	4,895	399	8,189
	" 3	3.790	17,124	4,399	768	1,449	12,725	10,508	2013	9,803
	" 1	5.617	25,382	6,940	1,047	1,880	18,442	15,515	4214	12,004
Exp. 240 Steer 57	Period 14									
	" 10	—	—	—	—	—	—	—	—	7,838
	" 2	1.700	7,596	2,072	387	745	5,524	4,392	223	8,061
	" 4	3.085	13,778	3,629	623	1,272	10,139	8,243	760	8,598
	" 6	4.612	20,608	5,484	874	1,781	15,124	12,469	2049	9,887
	" 8	6.233	27,851	8,029	1,123	2,335	19,822	16,363	4377	12,215
	" 8	8.057	36,026	10,880	1,360	2,930	25,146	20,855	6200	14,038

\* Corrected to represent the standard day and uniform live weight.

Steer 60	Fasting	—	—	—	—	—	—	—	—	—	—	—
Period 15												
" 11	½ maintenance	1.681	7,511	2,128	374	738	5,383	—	—	—	—	7,034
" 1	Maintenance	2.828	12,630	3,461	562	1,061	9,169	7,545	559	7,593	—	7,593
" 3	1½ times maintenance	4.237	18,933	5,466	743	1,483	13,467	11,241	1161	8,195	—	8,195
" 5	2 times maintenance	5.704	25,488	7,484	1,017	2,086	18,004	14,902	2466	9,500	—	9,500
" 7	2½ times maintenance	7.520	33,602	10,337	1,280	2,518	23,265	19,467	4504	11,538	—	11,538
" 9	3 times maintenance	9.489	42,429	13,959	1,500	3,075	28,470	23,896	6932	13,966	—	13,966
									8666	15,700	—	15,700

\* Corrected to represent the standard day and uniform live weight.

clusively in quantities of 50, 100, 150, and 200 grams, and measured also the basal metabolism of his subject. He found that the increase in heat production above the basal value was 19 calories, or 7.7 per cent, on the 50 gm. dose; 53 calories, or 13.1 per cent, on the 100 gm. dose; 118 calories, or 24.0 per cent, on the 150 gm. dose; and 171 calories, or 25.5 per cent, on the 200 gm. dose. Accordingly, the heat increment per 50 gm. of casein ingested at the different levels of feeding, from the lowest to the highest, was 19 calories, 27 calories, 39 calories, and 43 calories. When these data are plotted, a curve of heat production is obtained which is very similar to the curve of heat production (Fig. 1) derived at this Institute.

Williams, Riche and Lusk (4) recalculated the values given by Gigon above, by relating the increase in heat production not to the increase in protein ingested but to the increase in protein metabolism, and obtained the following heat-increment values per 100 calories of protein katabolized at the different levels of feeding:

50 gm. casein . . . . .	67 cal.
100 " " . . . . .	56 "
150 " " . . . . .	83 "
200 " " . . . . .	74 "
Average . . . . .	70 "

These values do not show a progressive increase with rise in plane of nutrition, as is shown by Gigon's original heat-increment values, which indicates that the katabolism of body protein below maintenance includes a factor of specific dynamic action (or waste heat of utilization). This factor may account, to a large extent, for the relatively low heat-increment values of the protein at the low planes of nutrition as obtained by relating the heat production to the food ingested.

In either case, however, it is apparent that

the specific dynamic action of protein is not a constant, but that it differs with the plane of nutrition at which it is determined—which is in harmony with the foregoing interpretation of the curve of heat production.

However this curve of heat production is explained, and whatever the ultimate causes of the contributing phenomena, it may be considered as the resultant of the combination of individual curves representing the metabolism of protein, carbohydrate, and fat; and it is the purpose of the present paper to analyze the curve of heat production by resolving it into these components.

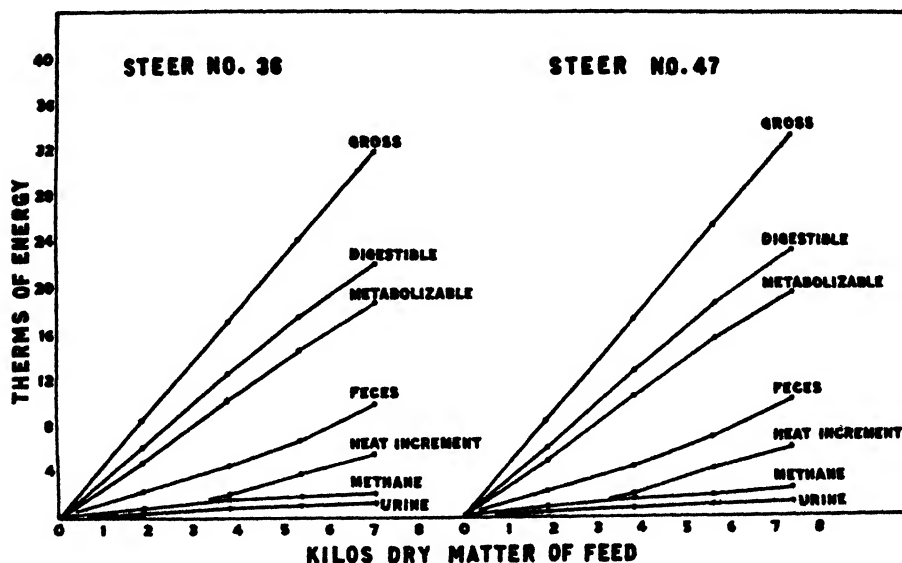


FIG. 3. The partition of the energy of the feed of cattle at different planes of nutrition.

#### PARTITION OF THE ENERGY OF THE FEED AND THE HEAT PRODUCTION OF CATTLE AT DIFFERENT PLANES OF NUTRITION

Figures 3 and 4 represent the partition of the gross feed energy, at the several planes of nutrition, between energy of digestible and of metabolizable nutrients, energy of urine, feces and methane, and energy expense of feed utilization—that is, heat increment.

In the experiments under discussion, no determinations were made of the oxygen consumption. Consequently no respiratory quotients were available for the determination of the proportions of the protein, carbohydrate and fat katabolized. It was found possible, however, to compute the energy of katabolism of these nutrients, as well as the energy of transformation of carbohydrate into fat, from the values determined for the production of

heat, urinary nitrogen, carbon dioxide, and methane. This computation, the results of which are presented in Table II, was accomplished as follows:

The energy and carbon dioxide resulting from the protein katabolized were computed from the urinary nitrogen by the use of Loewy's (5) factors (26.51 cal. and 4.75 liters  $\text{CO}_2$ , per gram of nitrogen). These quotas subtracted from the total heat production and total  $\text{CO}_2$  production, respectively, leave the non-protein energy and non-protein  $\text{CO}_2$ .

The  $\text{CO}_2$  of fat katabolism was then computed by means of the equation (liters non-protein  $\text{CO}_2$  + liters  $\text{CO}_2$  from  $\text{CH}_4$  -  $x$ )  $5.047 + 6.628x = \text{non-pro}$

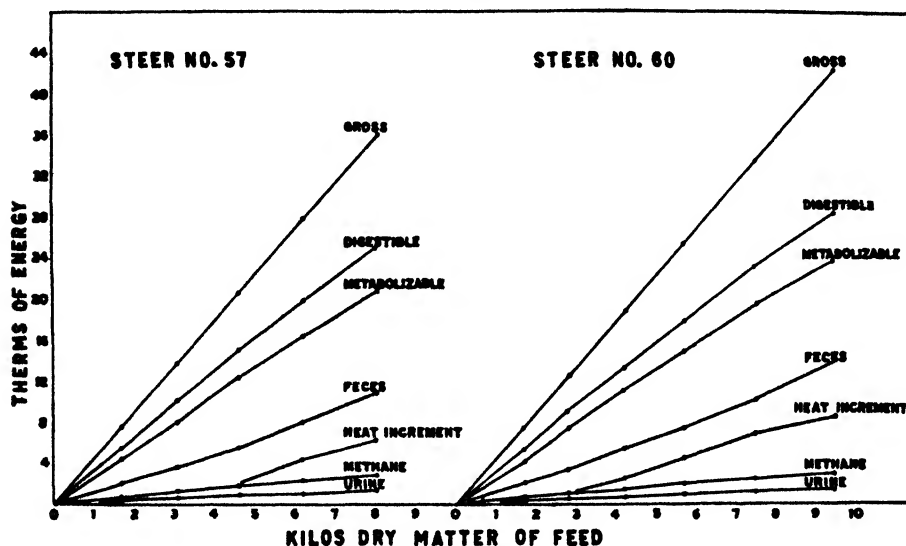


FIG. 4. The partition of the energy of the feed of cattle at different planes of nutrition.

tein Calories + Calories from  $\text{CH}_4$ , in which  $x$  represents liters of  $\text{CO}_2$  from fat katabolism; the figure 5.047 represents the calorific equivalent of a liter of  $\text{CO}_2$  from carbohydrate oxidation; and the figure 6.628 represents the calorific equivalent of a liter of  $\text{CO}_2$  from fat oxidation.

The addition of the  $\text{CO}_2$  from the oxidation of  $\text{CH}_4$  to the non-protein  $\text{CO}_2$ , and of the energy equivalent of the  $\text{CH}_4$  to the non-protein energy, is based on the principle evolved by Andersen (6) which places the metabolism of carbohydrate in a ruminant on a par with that in other species in which the carbohydrate katabolism is complete.

The calories from fat katabolism were then obtained by multiplying the liters of  $\text{CO}_2$  from fat oxidation by 6.628.

The energy of carbohydrate katabolism in the submaintenance periods is the difference between non-protein energy and energy from fat oxidation.

TABLE II  
COMPUTATION OF THE ENERGY OF PROTEIN, FAT AND CARBOHYDRATE KATABOLISM AND OF THE ENERGY OF TRANSFORMATION  
OF CARBOHYDRATES INTO FAT AT DIFFERENT PLANES OF NUTRITION

Experiment, animal and period No.	Urinary N	Prot. energy N $\times$ 26.51	Prot. CO <sub>2</sub> N $\times$ 4.75	Total heat prod. uncor. to stand. day	Total CO <sub>2</sub>	Non- prot. energy	Non- prot. CO <sub>2</sub>	CO <sub>2</sub> equiv. to CH <sub>4</sub>	Energy of CH <sub>4</sub>	CO <sub>2</sub> of fat synthe- sis*	Energy of fat synthe- sis	CO <sub>2</sub> of fat katab. <sup>†</sup>	Energy of fat katab.	Energy of carboh. katab.
	Grams	Cals.	L.	Cals.	L.	Cals.	L.	L.	Cals.	L.	Cals.	L.	Cals.	Cals.
Exp. 238 Steer 36 (Fasting)	12	1119	200	7651	1188	6532	988	—	—	—	—	978	6482	50
	6	1111	199	8156	1531	7045	1332	87	831	—	—	452	2996	4049
	8	1249	224	9840	2041	8591	1817	154	1481	—	—	79	524	8067
	4	1880	337	11854	2549	9974	2212	192	1842	80	87	—	—	9887
	2	2211	396	13888	3013	11677	2617	226	2168	127	138	—	—	11539
Steer 47 (Fasting)	11	991	178	7396	1146	6405	968	—	—	—	—	961	6370	35
	5	1095	196	7755	1463	6660	1267	86	827	—	—	416	2757	3903
	7	1270	228	9383	1975	8113	1747	151	1449	4	4	—	—	8109
	3	1967	352	11693	2572	9726	2220	196	1880	149	162	—	—	9564
	1	2309	414	13536	3010	11227	2596	255	2445	181	197	—	—	11030
Exp. 240 Steer 57 (Fasting)	14	1039	186	7482	1158	6443	972	—	—	—	—	972	6442	—
	10	1052	189	7939	1456	6914	1267	78	745	—	—	551	3652	3262
	2	1265	227	7909	1652	6644	1425	133	1272	—	—	34	225	6419
	4	1190	213	9493	2089	8303	1876	186	1781	82	89	—	—	8214
	6	1599	286	11857	2702	10258	2416	244	2335	210	229	—	—	10029
	8	2101	377	14408	3294	12306	2917	307	2930	262	286	—	—	12020

Steer 60 (Fasting)	15	43.2	1145	205	6904	1074	5759	869	—	—	—	—	869	5759	—
11	39.1	1037	186	7476	1384	6439	1198	869	77	738	—	—	469	3109	3330
1	40.4	1071	192	7253	1493	6182	1301	1198	111	1061	—	—	74	490	5692
3	39.8	1055	189	8821	1909	7766	1720	1301	155	1483	54	59	—	—	7707
5	49.7	1318	236	11157	2474	9839	2238	1720	213	2086	112	122	—	—	9717
7	66.5	1763	316	13976	3147	12213	2831	2238	264	2518	225	245	—	—	11968
9	88.4	2343	420	16133	3714	13790	3294	3294	322	3075	350	382	—	—	13408

\* Let  $x$  = liters  $\text{CO}_2$  resulting from the conversion of carbohydrates into fat; then (non-protein  $\text{CO}_2 + \text{CO}_2$  from  $\text{CH}_4 - x$ )  $5.047 + 1.09x$  = non-protein energy + energy of  $\text{CH}_4$ . Solve for  $x$ .

† Let  $x$  = liters  $\text{CO}_2$  of fat katabolism; then (non-protein  $\text{CO}_2 + \text{CO}_2$  from  $\text{CH}_4 - x$ )  $5.047 + 6.628x$  = non-protein energy + energy from  $\text{CH}_4$ . Solve for  $x$ .

The energy of transformation of carbohydrates into fat was computed by the use of the equation (non-protein  $\text{CO}_2 + \text{CO}_2$  from  $\text{CH}_4 - x$ )  $5.047 + 1.09x$  = non-protein energy + energy of  $\text{CH}_4$ , in which  $x$ , represents liters of  $\text{CO}_2$  resulting from the conversion of carbohydrates into fat; the figure 5.047 is the calorific equivalent per liter of  $\text{CO}_2$  from carbohydrate oxidation; and the figure 1.09 is the calorific equivalent (7) of a liter of  $\text{CO}_2$  resulting from the synthesis of fat from carbohydrates.

The energy of carbohydrate katabolism in the supermaintenance periods is the difference between the non-protein energy and the energy of fat synthesis.

These calculations are based upon the well established factors commonly employed in indirect calorimetry. Adequate discussions may be found in Lusk's Science of Nutrition (7), and in other reference works, of the derivation of the factors for computing protein energy and protein  $\text{CO}_2$ ; of the caloric equivalents of the  $\text{CO}_2$  resulting from the oxidation of carbohydrates and fat; and of the heat liberated in the conversion of carbohydrates into fat.

The method of indirect calorimetry, as it applies to cattle, including the principle of Andersen for accounting for the methane fermentation, has been critically discussed by Forbes and associates (8).

The whole process of resolving the heat production into its components, which has been employed in this paper, is in reality only the reverse of the process of computing the total heat production by indirect calorimetry, as adapted by Andersen on account of the extensive production of methane in the ruminant alimentary tract.

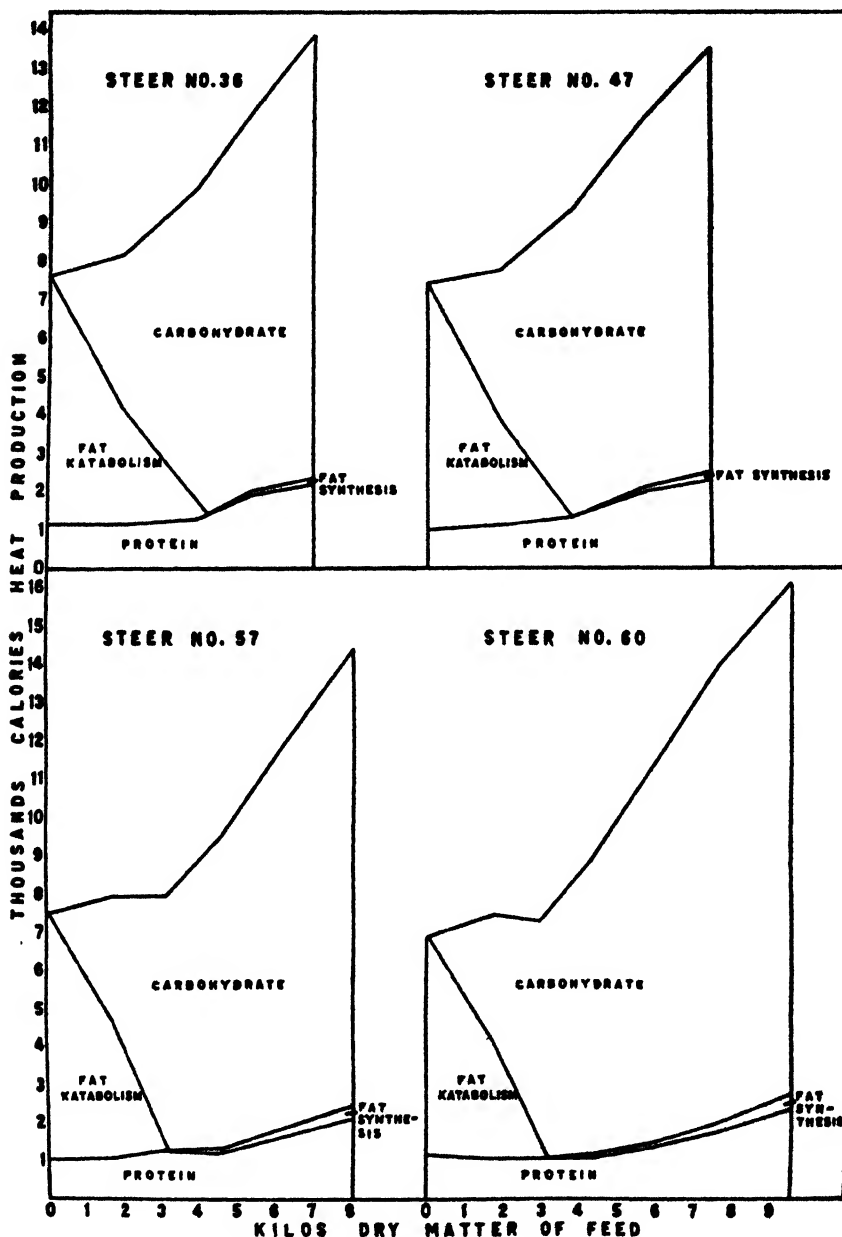


FIG. 5. The partition of the heat production of four steers at planes of nutrition between fast and two to three times the maintenance requirement of energy.

The elements of error in the factoring of the heat production, therefore, are exactly the same as obtain in the computation of the heat production by Andersen's modification of the standard respiratory quotient procedure.

As to the element of error in the starting point of this computation, that is, the directly determined heat production, Forbes and associates (8) compared 18 such measurements of the heat production of cattle with parallel computations of the heat production by the respiratory quotient method, as adapted by Andersen, and found that all of the computed values were between 98 and 104.8 per cent of the directly observed values, the average being 102.1 per cent, with a standard deviation of  $2.15 \pm 0.25$  per cent.

Attempts have been made to derive further confirmation of the findings here reported, from the extensive calorimetric data on record at this Institute. A study of these materials shows unmistakably that they conform to the principles herein set forth, but they are not cited in detail since they are less perfectly adapted to the demonstration of these matters than are the results upon which this paper is based.

The contributions to the total heat production of heat from protein, from fat, and from carbohydrate, at the different planes of nutrition, are presented in Table II and in Figure 5, the separate quotas being parts of the directly observed heat production without reference either to the differences in the live weight of the animals, in the several experimental periods, or to the differences in the intervals of time spent by the animals in the standing and the lying positions.

In this relation it is significant that the sequence of the experimental periods (as already explained) was purposely arranged otherwise than in natural serial order, which determined the fact that the live weights, and therefore the maintenance quotas, also differed otherwise than in natural serial order. On this account the curves of uncorrected heat production in Fig. 5 are not so nearly symmetrical as are the curves in Figs. 1 and 2, which are based on the heat production corrected to represent uniform live weight and maintenance requirement.

The same fundamental data as referred to in the foregoing paragraph, computed to percentages of the total heat production, are presented in Table III, and in Figs. 6 and 7.

The energy of protein katabolism with the four animals constitutes a nearly constant proportion of the total heat production.

The values for energy of fat katabolized during periods of fasting (Table II, 13th column of data) are approximately the same as the values for non-



protein energy (6th column of data), in experiment No. 238, and exactly the same in experiment No. 240.

This signifies that in these periods the animals were in a state of true fast, with no oxidation of carbohydrate. The fasting heat measurement with steer No. 36 was during the fifth, sixth, and seventh days; with No. 47 during the fourth, fifth and sixth days; and with Nos. 57 and 60 during the fourth day, of fast—thus signifying that the equations and factors used in the computation are satisfactory.

TABLE III  
PERCENTAGE CONTRIBUTIONS OF HEAT FROM PROTEIN, FAT AND CARBOHYDRATES TO  
THE TOTAL HEAT PRODUCTION AT DIFFERENT PLANES OF NUTRITION

Experiment, animal and period No.	Plane of nutrition	Energy of protein katab.	Energy of carbohy- drate katab.	Energy of fat katab.	Energy of fat synthesis
Exp. 238					
Steer 36					
Period 12	Fasting	14.6	0.7	84.7	—
" 6	$\frac{1}{2}$ maintenance	13.6	49.6	36.7	—
" 8	Maintenance	12.7	82.0	5.3	—
" 4	$1\frac{1}{2}$ times maintenance	15.9	83.4	—	0.7
" 2	2 times maintenance	15.9	83.1	—	1.0
Steer 47					
Period 11	Fasting	13.4	0.5	86.1	—
" 5	$\frac{1}{2}$ maintenance	14.1	50.3	35.6	—
" 7	Maintenance	13.5	83.4	—	—
" 3	$1\frac{1}{2}$ times maintenance	16.8	81.8	—	1.3
" 1	2 times maintenance	17.1	81.5	—	1.3
Exp. 240					
Steer 57					
Period 14	Fasting	13.9	—	86.1	—
" 10	$\frac{1}{2}$ maintenance	13.3	40.7	46.0	—
" 2	Maintenance	16.0	81.2	2.8	—
" 4	$1\frac{1}{2}$ times maintenance	12.5	86.6	—	0.9
" 6	2 times maintenance	16.9	81.2	—	1.9
" 8	$2\frac{1}{2}$ times maintenance	14.6	83.4	—	2.0
Steer 60					
Period 15	Fasting	16.6	—	83.4	—
" 11	$\frac{1}{2}$ maintenance	13.9	44.5	41.6	—
" 1	Maintenance	14.8	78.4	6.8	—
" 3	$1\frac{1}{2}$ times maintenance	12.0	87.3	—	0.7
" 5	2 times maintenance	11.8	87.1	—	1.1
" 7	$2\frac{1}{2}$ times maintenance	12.6	85.6	—	1.8
" 9	3 times maintenance	14.5	82.2	—	2.4

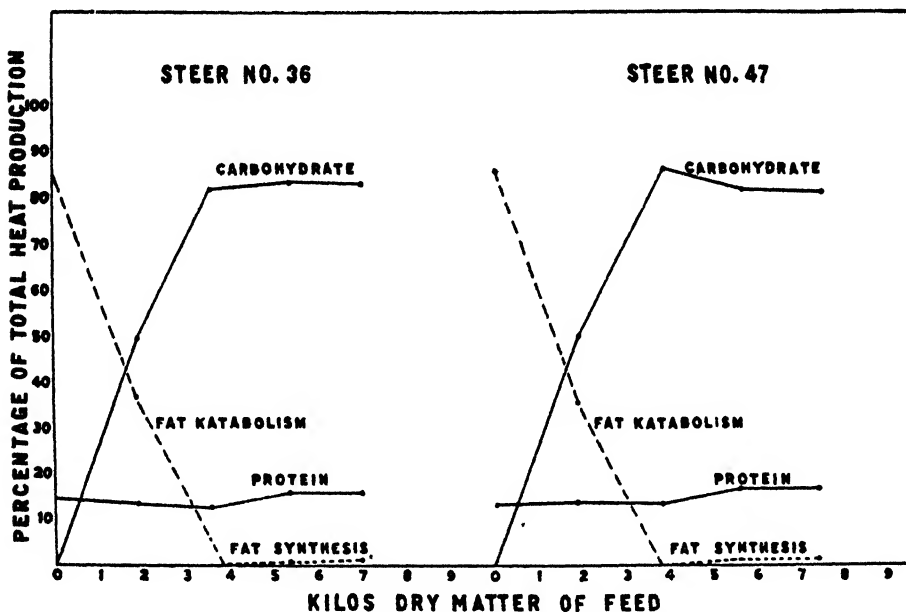


FIG. 6. The contributions of protein, fat, and carbohydrate to the heat production of cattle at different planes of nutrition.

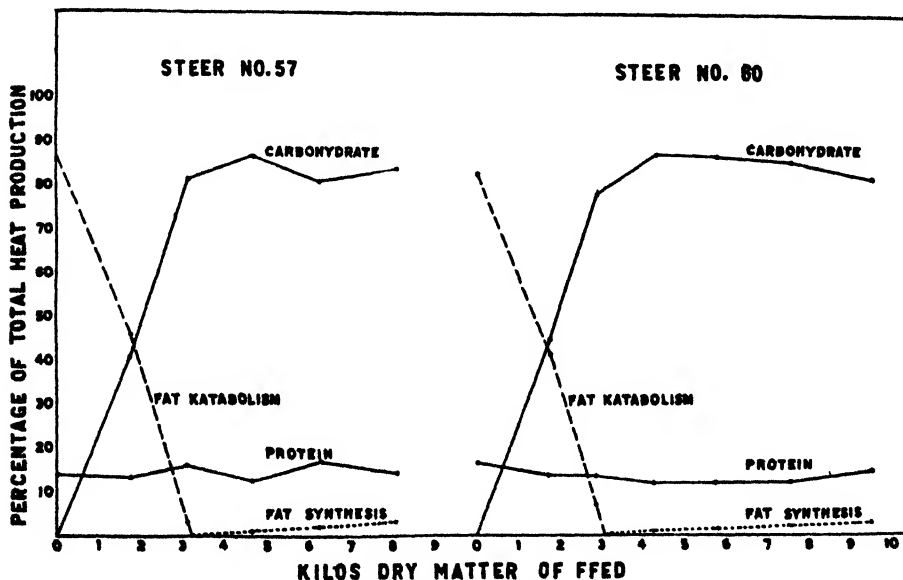


FIG. 7. The contributions of protein, fat, and carbohydrate to the heat production of cattle at different planes of nutrition.

It is shown in Table III that during fasting, 83.4 to 86.1 per cent of the heat is produced from fat, and the remainder from protein.

At half-maintenance, fat and carbohydrate contribute nearly equally to the heat production; at maintenance the heat production from fat disappears, while the contribution from carbohydrate is greatly increased, and approximately equals the heat production from fat during fasting.

Above maintenance a small proportion of the heat production results, without oxidation, as a by-product of the synthesis of fat from carbohydrate. This fraction naturally increases with rise in the plane of nutrition, but remains a minor factor; while the proportion derived from carbohydrate remains approximately the same as at maintenance.

The composition of the ration was the same in all periods in which the animals received food; the differences in the proportions of protein, fat and carbohydrate katabolized below maintenance, therefore, are results of the replacement of body nutrients by food nutrients, with rise in the plane of nutrition from fast to maintenance; and above maintenance there is comparatively little variation in these proportions. Presumably this factor of difference in the proportions of the nutrients of the different kinds which are metabolized at the different planes of nutrition contributes in some degree to the curve of heat production in relation to food consumption, but exact information as to the extent of this component is lacking.

#### SUMMARY

The curve of heat production of cattle, between points of fasting and three times the maintenance requirement, is analyzed by computation and graphic representation of the contributions of heat from the katabolism of protein, fat, and carbohydrate.

The derivation of heat from body substance and from food nutrients is shown, as also is the energy expense of synthesis of fat.

The contribution from protein is about the same proportion of the total heat production at all planes of nutrition.

The contribution of fat is 83.4 to 86.1 per cent of the total heat production at fasting, and apparently falls to zero at maintenance.

The proportionate contribution of carbohydrate between fasting and maintenance is approximately the complement of that of fat. From maintenance to three times maintenance the proportionate contribution of carbohydrate does not vary greatly.

The energy cost of fat synthesis is a minor factor, above maintenance.

The causes of the curvature of the line representing the relation of the heat production to the food consumption of cattle, with rise in the plane

of nutrition from fasting to full feed, seem to be virtually as suggested in former publications, 1.—the increase in intermediary metabolism resulting from increasing concentration of circulating metabolites, 2.—the decrement of waste heat of utilization of body nutrients, between fasting and maintenance, 3.—changes in the proportions of protein, fat, and carbohydrate katabolized, and probable (but unestablished) differences in the specific dynamic effects of these different katabolized nutrients, 4.—the energy expense of synthesis of fat from carbohydrate, above maintenance, and 5.—the decreasing metabolizability of the food at the higher planes of nutrition, 6.—the heat of fermentation of carbohydrate nutriment, and 7.—the physical work of food utilization.

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# IS THE VITAMIN B CONTENT OF MILK UNDER PHYSIOLOGICAL CONTROL?\*

By

FRANK L. GUNDERSON AND H. STEENBOCK

*(From the Department of Agricultural Chemistry, University  
of Wisconsin, Madison.)*

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INVESTIGATIONS bearing on the vitamin B content of milk may be said to have started with the work of Hopkins when he found in 1906 and 1907 that 2 cc. of milk sufficed for a young rat's need for accessory food factors. These results were published in 1912 by Hopkins (1). Osborne and Mendel (2) in 1911 reported success with rats which were used in determining the comparative nutritive value of various proteins when their synthetic diets contained protein-free milk. Later (3) they reported "a considerable degree of success in the absence of milk protein as well as of the hypothetical organic 'hormones', etc., present in the milk." Hopkins and Neville (4) were not so optimistic after their feeding trials. They demonstrated failure of growth unless their synthetic rations were supplemented with 2 cc. of milk per diem. This amount produced practically normal growth. Osborne and Mendel were able, after continuing their experiments over a longer period of time (5), to substantiate these results except with respect to the amount of milk required. A supplement of 2 cc. of milk daily rarely sufficed to allow the animals to make more than a very slight gain in weight. They state (p. 542) "It scarcely seems plausible that milk from different sources, even though produced from cows on unlike feeds, should account for the wide variation noted; in fact, we have used samples from several local sources, always with the same result," and (p. 541) "Not until at least 16 cc. of fresh milk per day were supplied along with the food mixture was anything approaching a normal rate of growth secured." In 1920 Osborne and Mendel (6) duplicated the ash of Hopkins' synthetic ration as closely as they could and obtained the same results as before. Even 2 cc. of unpasteurized milk when produced by cows on pasture supported but little, if any, growth in the young rats. Thereupon, Hopkins (7) reported further experiments in an attempt to clear up the discrepancies, anticipating possible differences in the nutritive value of milk or the response of the rats with change of season. Some of these experiments were carried out with goat's milk and others with cow's milk. Some of his re-

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sults obtained during the winter were, as stated by him, "frankly disappointing," but the favorable effect of 2 cc. of milk was nevertheless unmistakable and all of his other experiments proved as stated by him (p. 723) "that given the right conditions my original observations can be repeated." Stammers (8) obtained results, which, while they did not disprove Osborne and Mendel's conclusions, threw additional weight of evidence in favor of Hopkins' findings. Coward and Clark (9) obtained normal growth on 8 cc. Larger or smaller amounts were apparently not fed. These findings taken all together leave the situation unexplained.

In 1920, impressed by the necessity for clarifying the situation, we initiated a series of experiments in which we tested the influence respectively of breed, pasture, early lactation, late lactation, and the effect of increasing the vitamin B<sup>1</sup> content of the ration. The results of these experiments are shown in Chart 1.

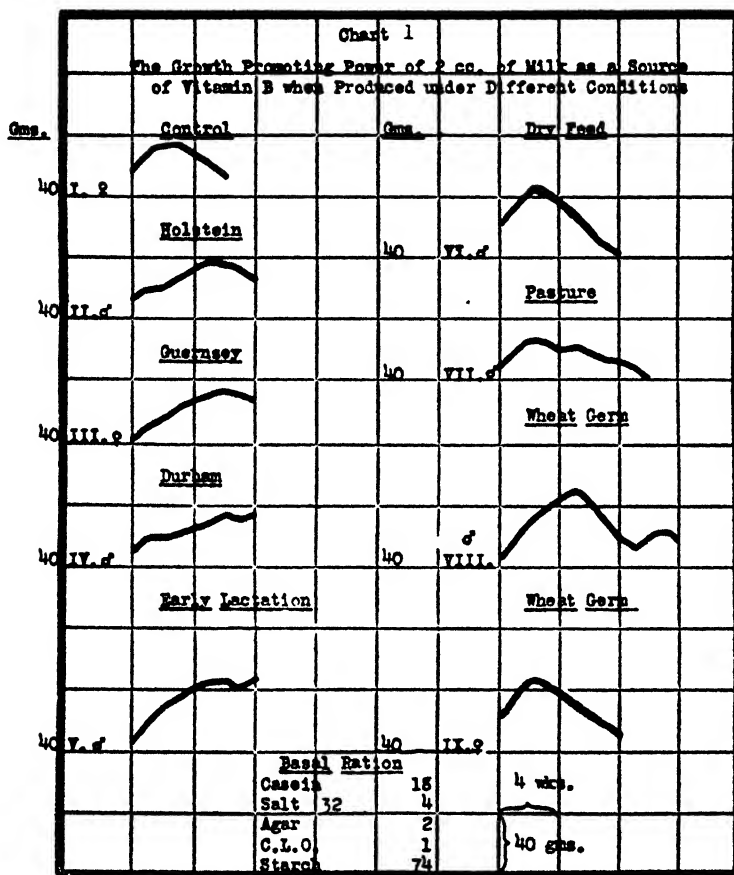
The rats used for these feeding trials were males and females weighing approximately 40 to 60 gms. at 23 to 27 days of age. They were kept individually on wood shavings in wire cages measuring 12×24×20 inches. Water and basal ration were fed *ad libitum*. The milk was pipetted off in small glass dishes. The basal ration consisted of; alcohol-extracted and heated casein 18 per cent, salts 32 (10) 4 per cent, agar 2 per cent, cod liver oil 1 per cent, and alcohol-extracted, cooked starch 74 per cent.

The data shown in Chart 1 present the results obtained with only one rat out of the 4 in each group selected for its representative performance. Graph 1 shows the growth obtained on the basal ration; the others show the result of supplementing the basal ration with 2 cc. of milk obtained from the various sources.

Graphs II, III, and IV, were obtained with milk produced by cows of the Holstein, Guernsey, and Durham breeds respectively. We considered it worth while to test out different breeds because of the possibility that Hopkins might have obtained his data from cows of the Durham breed. The cows used by us received a grain mixture composed of; yellow maize 3 parts, oats 3 parts, bran 3 parts, oil meal 1 part, and cotton seed meal 1 part, together with hay and silage. The Holstein cow had been milked continuously for 15 months and at the time of the experiment was producing 9 lbs. of milk daily. The Guernsey cow had been milked for 8 months and was producing 8 lbs. daily. Exact data on the Durham cow could not be obtained. She was suckling a calf on another experimental project and was producing barely enough for its needs.

<sup>1</sup> In this paper the term vitamin B refers to the ensemble of factors formerly thought of as a single entity.

Graph V shows results obtained with milk from a recently freshened Holstein cow during the months of October and November. She was giving 27 lbs. of milk daily when the experiment was started one week after parturition. It is evident from comparison with Graph II, which was obtained with milk from the Holstein cow, 15 months after parturition, that the stage of lactation was no factor in vitamin B secretion. Furthermore,



these graphs in comparison with Graphs III and IV show that the vitamin B content, at least under the conditions of our experiment, was not subject to variations with breed.

Graphs VI and VII were obtained with milk from a cow which was kept on dry feed during the months of May and June and then changed to pasture during July and August. She was of the Holstein breed and was producing about 40 lbs. of milk daily. During the dry feed period, she received daily 12 lbs. of alfalfa hay, 30 lbs. of corn silage and 12 lbs. of a



grain mixture which contained constituents mixed in the proportion; maize 4 lbs., oats 8 lbs., and oil meal 3 lbs. During the pasture period, she received 18 lbs. of the grain mixture and fresh alfalfa in her stall during the daytime and mixed pasturage at night. The rat experiments were started 6 weeks after parturition. From the results obtained, already referred to by Hart (11), it is evident that fresh succulent feed had no effect on the secretion of vitamin B. This confirms observations of Osborne and Mendel (6).

Graphs VIII and IX show the results of our attempt to increase the vitamin B content of milk by definitely increasing its amount in the feed. The source of the vitamin B was wheat germ which has been reported by Bell and Mendel (12) to be 10 times as rich in vitamin B as the wheat kernel. Our sample was probably not quite so potent because it was somewhat contaminated with bran. The cows used were of the Guernsey and Holstein breeds respectively. The former had freshened 4 months previously and was giving 14 lbs. of milk daily. The latter had freshened one month before and was giving 12 lbs. daily. The Guernsey received a mixture of mixed hay 10 lbs., maize silage 30 lbs., and grain 7 lbs. This latter was composed of equal parts whole corn meal and wheat germ. The Holstein cow received 10 lbs. of wheat germ and 10 lbs. of clover hay. Feeding of these rations was started at least 4 weeks before milk was used for the experiments.

The graphs produced herewith show very clearly that in spite of replacing part of the grains with wheat germ, absolutely no effect on the growth of the rats resulted. That the rats were in condition to respond to additions of vitamin B was demonstrated repeatedly by supplementing their basal ration plus 2 cc. of milk daily with an alcohol extract of wheat germ corresponding to 6 gms. of wheat germ at the close of the trials. These results have already been briefly referred to (13).

It is to be admitted that our technic of determining vitamin B in 1921 was not so satisfactory as it is today. In the first place, it is to be remembered that we were always dealing with the entire ensemble of vitamin B's and, furthermore, it was not until 1923 that we (14) pointed out the necessity of keeping rats on screens to reduce coprophagy to a minimum. As already stated, the rats in the preceding experiments had been kept on shavings. McCollum, Simmonds, and Becker (15) spoke disparagingly of the innovation of keeping rats on screens, pointing out that with frequent cleaning of cages, it is not necessary. They apparently overlooked the fact that two years before their paper was published, we had reported cleaning our cages daily, but this we found to be less effective and much

more laborious. At present, the use of screens is generally accepted as good technic in feeding experiments.

It is, however, scarcely probable that Hopkins obtained his pronounced growth effects in consequence of coprophagy. As a matter of fact, we are led to believe that he practically eliminated this factor because he states (1) that he used wire cages on legs 4 inches high to prevent consumption of filter paper placed below and that the greater part of the feces fell through the cage on to the paper beneath. One gathers the impression that his screen bottoms were probably of rather smaller mesh than those used by us. He, furthermore, emphasizes the necessity of cleanliness.

There are, however, other factors besides coprophagy which demand consideration. In analyzing data obtained with rats on low vitamin B diets, one occasionally notices unusual growth in certain individuals or groups. These performances cannot be explained on the basis of pre-experimental storage of vitamin B, because it is well known that such storage does not take place (16). These, likewise, are scarcely to be explained by difference in consumption of excreta, although certain individuals and correspondingly, therefore, certain groups, are sometimes unusually adept at coprophagy. Scheunert and Schieblich (17) found that vitamin B was synthesized by certain bacteria found in the intestine. Heller, McElroy, and Garlock (18) produced evidence of bacteriological synthesis of vitamin B in the intestine of rats. Fridericia *et al.* (19) provided actual evidence for possible dispensability of vitamin B in the ration by what they termed refection. This they attribute to a probable synthesis of vitamin B by certain organisms which develop in the intestinal tract under certain experimental conditions. Roscoe (20) observed the phenomenon of refection independently at about the same time. Kon and Watchorn (21) confirmed these observations. Roscoe (20) reported that the rats in which it occurred were all piebald. Albino rats kept in the laboratory at the same time were not affected. Pierce, Osgood, and Polansky (22) reported a difference in the rapidity with which their strain of rats responded to lack of vitamin B in comparison with strains purchased from an animal dealer. However, they observed that their rats, which were piebald, were not so sensitive as the albino. Even though there is no unanimity of conclusions on resistance of different strains, the question may well be raised whether it is possible that Hopkins had refection in his milk fed animals but not in his controls.

That synthesis of vitamin B can occur through action of micro-organisms in the rumen of the cow, was suggested by Theiler, Green, and Viljoen (23) and has been demonstrated by Bechdel and co-workers (24,

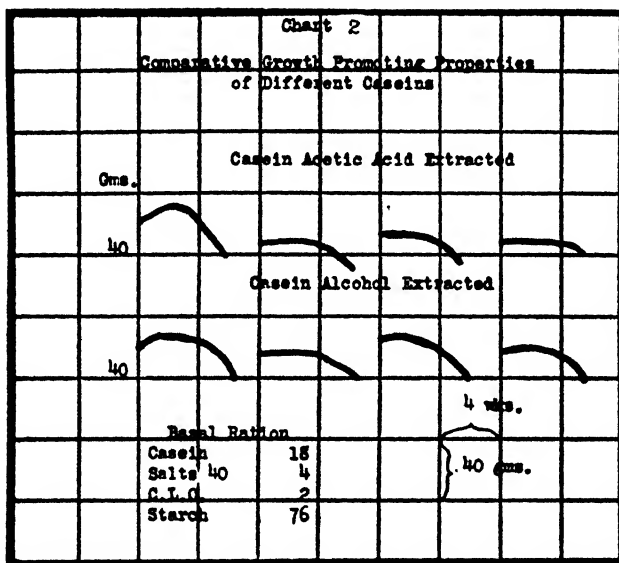
25, 26). The last named not only demonstrated that the amount synthesized was sufficient to maintain normal growth in young heifers, but also that it was sufficient to produce milk of a vitamin B content normal or only slightly less than normal. However, continued lactation was not accomplished,—disaster supervened after one or two weeks. It should be mentioned that such a synthesis of vitamin B may not always occur in the ruminant. Hughes, Fitch and Cave (27) for instance, have reported low vitamin B values in cow's milk produced on a low vitamin B ration. Kennedy and Dutcher (28) made similar observations.

Reference should also be made to the recent noteworthy findings of Evans and Lepkovsky (29) who demonstrated that an increase in the fat content of the diet decreased the rats' requirements for vitamin B. This they associated with the presence of certain fatty acids and mixtures of fatty acids in the glycerides. It is admitted that our rations were low in fat, carrying only 1 per cent of cod liver oil and the fat of the milk supplement. Hopkins (1) used a basal ration containing 12.4 per cent of lard, but Osborne and Mendel (5) used a basal ration which contained 18 per cent of lard and 9 per cent of butter fat. It is, therefore, scarcely possible that the difference in effectiveness of the milk supplements can be explained by difference in intake of fatty acids, even in full recognition of the variability in fatty acid content of fat produced under different conditions.

Within the last several years we have had occasion to reinvestigate the remarkable constancy in the vitamin B content of milk, as used in our laboratory, not to mention the discrepancy in our results compared with those of Hopkins. We carried out these new experiments with both cow's and goat's milk, preventing coprophagy by the use of screen cage bottoms and giving full recognition to the existence of other vitamins in the vitamin B complex. The cows' milk was obtained from the university herd consisting mostly of cows of the Holstein breed with some of the Jersey, Guernsey, Ayrshire, and Brown Swiss breeds as well. The goats were pure bred Toggenbergs or grades.

Male rats weighing approximately 60 gms., as raised in our laboratory, were used exclusively for the tests. They were fed a basal ration of casein 18 per cent, salt 40 (30) 4 per cent, cod liver oil 2 per cent, cooked starch 74 per cent. Sometimes 2 per cent of agar was included in this ration, but we were inclined to question the necessity for the use of roughage under the conditions of our experiment. Substances other than milk, when tested for vitamin B, were introduced into the ration at the expense of an equal amount of starch. Milk was fed as a supplement to the entire diet.

The casein used in our rat rations was Argentine acid casein, which had been purified by extraction with water acidified with acetic acid for a week. In some cases we used the same commercial casein which had been purified by repeated extraction with hot alcohol for a week. Preliminary feeding tests, the results of which are shown in Chart 2, showed that it was immaterial which casein was used. In both instances the amount of growth obtained compared favorably with that reported by Palmer and Kennedy (31) with casein purified by a much more laborious procedure.

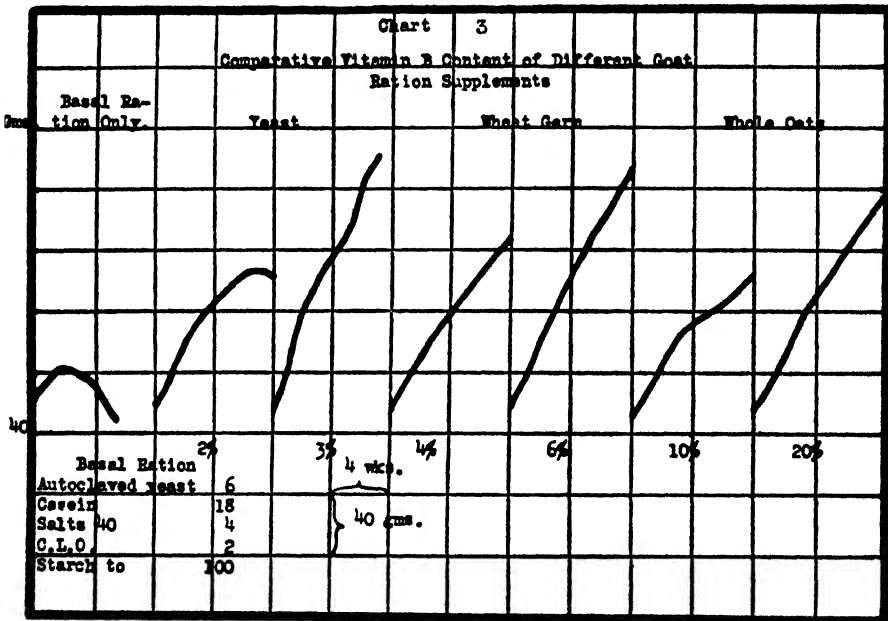


In one case we incorporated heated yeast in the rat ration to supply vitamin G. This was prepared by autoclaving dry yeast (obtained from the Northwestern Yeast Company) in a layer one inch thick in an enameled pan at 15 lbs. pressure for 2-1/2 hours, and then drying at 60 to 95° Centigrade. A test with four rats revealed the presence of only very small amounts of vitamin B when fed at a level of intake constituting 24 per cent of the ration. Three of the rats on this experiment died in from 5 to 7 weeks; on the basal ration alone, they died in from 5 to 6 weeks.

The goat rations were built up around a daily intake of 0.75 lb. of alfalfa and 1.5 lbs. of a grain mixture. This latter consisted of a basal mixture of 35 parts yellow corn meal, 30 parts wheat bran, 5 parts linseed oil meal, and 1 part of sodium chloride. This basal mixture was supplemented respectively with 30 parts of oats to make the "barn ration"; with 30 parts of wheat germ to make the "wheat germ ration," and with

15 parts each of yeast and oats to make the "yeast ration." Taking the daily ration, which amounted to 2.25 lbs. of hay and grain as a whole, the three rations differed from one another in that they contained respectively 20 per cent oats, 20 per cent wheat germ, and 10 per cent of dried yeast. We made numerous attempts to secure a larger percentage intake of wheat germ and yeast, but this tended to interfere with consumption.

In Chart 3, we present some results of tests designed to reveal the comparative vitamin B content of the oats, wheat germ, and yeast as used in our rations. In order to conserve space, only one rat of each group of four



was charted. Those charted were taken as being representative of the group. From their performance it appears that approximately 3 parts of yeast were equivalent in vitamin B content to 6 of wheat germ and 20 of oats. These amounts gave slightly better than normal growth, while 2, 4, and 10 parts, respectively, of the aforementioned constituents gave sub-normal growth of approximately equal intensity.

In a previous publication we (14) have stated that approximately 60 parts of a grain such as oats are necessary for normal growth. However, in those cases the grain was relied upon to furnish both vitamins G and B. It is now well known that at least the oat kernel is preponderatingly low in the former with respect to the requirements of rats (32). We accordingly supplied vitamin G in the present vitamin B assays of yeast, wheat germ,

and oats, in the form of autoclaved yeast. We did not use "equalized consumption" technic in these experiments, although in general we are committed to this procedure (33). The probability of error due to its non-use was not large, as revealed by our consumption data. With 2, 4, and 10 per cent of yeast, wheat germ, and oats respectively in the rations, the amounts consumed were 7.9, 10.4, and 8.9 gms.; with 3, 6, and 20 per cent of these materials the consumption was 9.9, 9.4, and 8.9 gms. respectively.

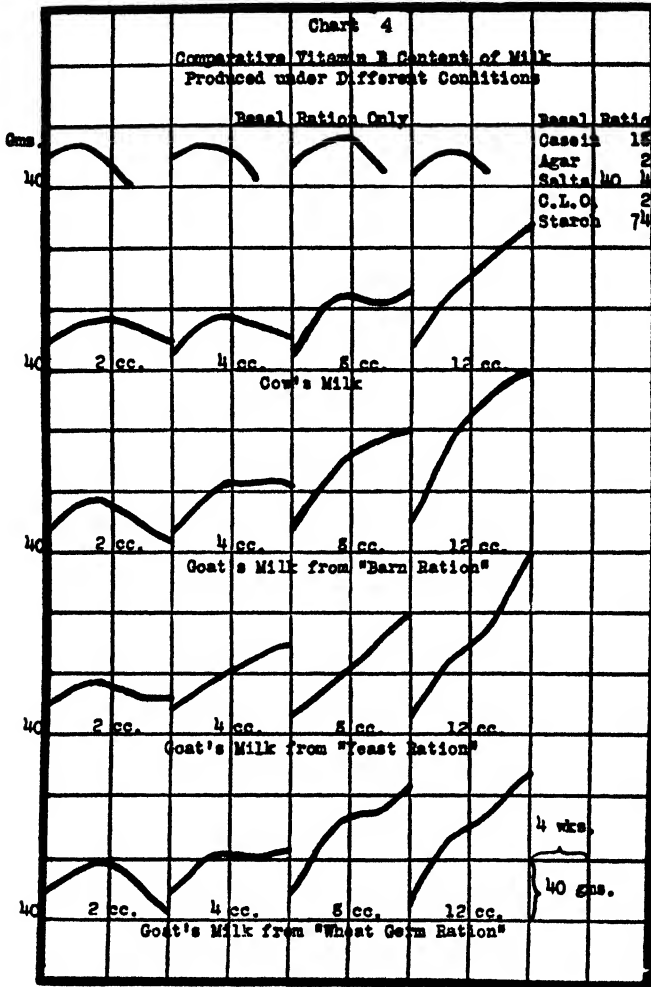
For the determination of vitamin B in milk, we used an improved form of our earlier technic, putting our stock male rats at approximately 60 gms. on screens, four in a group with individuals segregated. The milk was fed fresh, pipetted out in quantities of 2, 4, 8, and 12 cc. daily. The animals were weighed weekly.

Chart 4 shows the degree of growth failure which we obtained on our basal ration alone. Very little improvement resulted from a supplement of 2 cc. of cow's milk, and even 12 cc. failed to give normal growth. This substantiates our previous observations on cow's milk, but the present results were more regular, due to the use of screens. It will be noted that the basal ration which we used in these milk tests did not contain autoclaved yeast nor other sources of vitamin G, because it has been shown (36) that vitamin G is not the growth-limiting factor in milk.

Chart 4 also shows the results obtained on feeding goat's milk at the aforementioned levels of intake as produced on the three rations already described. The results are essentially of the same order as those obtained with cow's milk, but on the average are somewhat better. Again there was no change in the vitamin B content resulting from an increased intake in the ration. No exact numerical expression of the increased vitamin B consumption on the fortified rations can be made for the consumption varied considerably from day to day. The alfalfa employed in all of the goat rations was of the same lot but was not assayed for vitamin B. However, on the basis of the amount of vitamin B reported in alfalfa (34, 35), we may consider that it was approximately equal to that of whole cereal grains. Separate rat assays of the three goat rations indicate that the vitamin B content of the "yeast" and "wheat germ" rations was practically double that of the "barn ration." It seems safe to consider that the total vitamin B consumed by the goats was increased about two-thirds on each of the two fortified rations over that on the "barn ration."

Milk production of the goats varied from one to five pounds daily per individual. As a rule, the output approximated two pounds daily. Calculations to correlate the vitamin B intake of the goats with that secreted in the milk show that on the "barn ration" about fourteen per cent, and

on each of the fortified rations about nine per cent, of the consumed vitamin B occurred in the milk. In other words, the amount of vitamin B per unit of milk seems to remain unchanged when the dietary intake of vitamin B is stepped up from a reasonably good level to one about two-



thirds higher. Our experience with goat's milk also is definitely at variance with Hopkins' experience, because he (7) obtained nearly normal growth when he supplemented his basal ration with 5 cc. of goat's milk daily. We definitely failed to accomplish this result with 8 cc. but approached it with 12 cc.

Our failure to increase the vitamin B content of goat's and cow's milk

by unusual increases in the amount in the ration, stands in marked contrast to the observed effects of feeding rations markedly deficient in vitamin B. Andrews (37) in 1912 produced beriberi in 16 pups when these were nursed by women who had previously lost their infants from beriberi. Improvement in other infants was also observed with the substitution of less highly milled rice for the polished rice in the mother's diet. From these results it is apparent that the mother is less sensitive to impending deficiency than is the infant. In 1918 we encountered a similar situation in experiments with rats on a ration markedly low in vitamin B. We failed to notice any signs of impending disaster in the nursing mother, but observed that the young were seized with convulsions from which they failed to recover until they were put with a nursing female maintained on a normal ration (38, 39). In 1924, Sure (40) also observed that the vitamin B requirements of the rat are much greater for normal mammary function than for growth. Later, he (41) showed that they are at least 3 times as great and that the requirement of abnormally large amounts is due, at least in part, to the unusually large losses incurred in the mother's metabolism (42). Hartwell (43) reported that the lactating rat requires at least 4 times as much vitamin B as the growing animal. Evans and Burr (44) found that 5 times the usual intake of vitamin B in the form of yeast was required for normal nutrition of the young suckling rat. Macy, Outhouse, Graham, and Long (45), also found an increase in the vitamin B requirement of the rat during lactation. Daniels, Jordan, and Hutton (46) believe that the high mortality of nurslings of rats on milk is due not to low vitamin B intake, but rather to insufficient intake of calories. Sure (47) emphatically denied this.

In general, it may be said that there is no dearth of evidence to prove that the vitamin B content of milk can be reduced below normal, yet it must be remembered that the failure of experiments to produce growth in young rats may not always be due to poor quality of milk, but to a deficiency in quantity. Sure and Schilling (48), however, have reported poor growth in young rats with an abundance of curd in their stomachs. In the light of this, our observations that vitamin B cannot be increased above a certain amount, stand out as being all the more remarkable.

The sufficiency of the vitamin B content of milk, both human and cows', for normal nutrition of the infant has been widely discussed. Daniels and Byfield (49) obtained favorable results in infant feeding with the use of a wheat germ extract, but Daniels (50) was not so successful with brewer's yeast because it caused diarrhea as an apparent result of intestinal irritation. Bloxsom (51), however, advised the use of brewer's yeast as a sup-



plement to the diet of the infant from birth. Hoobler, Macy and Outhouse (52) believe that breast milk cannot be relied upon to supply the optimum amount of vitamin B for the infant. Hoobler (53) believes that every infant should have an addition of vitamin B to its milk diet, whether cow's or human. Dennett (54), in studying the value of a commercial preparation of vitamin B prepared from wheat germ, found it valuable for infant feeding with both cow's and mother's milk. It is, of course, entirely possible that in these instances the mother's milk was produced on a vitamin B-poor diet and, therefore, may have been abnormally low in vitamin B, but it is scarcely probable that the cow's milk was similarly impoverished.

In 1928 Macy, Outhouse, and Hunscher (55) pointed out that with their lactating human subjects the vitamin B content of milk varied approximately inversely to the production level. Later work from the same laboratories by McCosh, Macy, and Hunscher (56) confirms this opinion. These investigators found that supplementation of the maternal diet with 10 grams of yeast daily caused the occurrence in the milk of some additional factor which they concluded pointed to better utilization of the food of experimental animals. However, they add that "It is obvious that this substance is not an appetite stimulator; it is consequently improbable that it is the antineuritic vitamin." From their data we are unable to conclude whether or not the vitamin B content of the diet was optimal for the production of milk of normal vitamin B potency. However, it seems quite probable that the vitamin B level in the "pre-experimental" diets of the lactating human subjects of McCosh, Macy, and Hunscher was not particularly high. In a certain way, their general results with humans are analogous to our data on vitamin B as obtained with cows and goats.

Our findings that cow's milk and goat's milk cannot have their vitamin B content increased beyond the usual level emphasize the necessity for recognizing the definite limitation of cow's and goat's milk in normal human nutrition as claimed by others. It remains to be seen whether or not our findings and the findings of those who claim that cow's milk should be fortified with vitamin B for infant feeding will be supported by future investigations. Obviously, there are many possible variables which enter into the determination of such general relations, and in the face of everything Hopkins' outstanding conclusions need to be harmonized with those of others.

#### CONCLUSIONS

Hopkins' conclusions relative to the high concentration of vitamin B in cows' milk could not be confirmed. No essential differences were found

in the vitamin B content of milk produced by cows of the Holstein, Guernsey and Durham breeds. The period of lactation did not appear to have any influence. Milk produced on pasture and fresh green alfalfa did not contain any more vitamin B than milk produced on silage and dry feed, thus confirming results obtained by Osborne and Mendel. Increasing the vitamin B intake both absolutely and in relation to the amount of milk secreted had no discernible effect on the vitamin B content of cows' or goats' milk. It appears, therefore, that the maximum vitamin B content of milk is under definite physiological control.

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MAY, 1932

## THE EFFECTS OF LOW ENVIRONMENTAL TEMPERATURE UPON METABOLISM\*

## I. TECHNIC AND RESPIRATORY QUOTIENT

BY RAYMOND W. SWIFT†

*(From the Department of Vital Economics, University of Rochester, Rochester, N. Y.)*

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ALTHOUGH it has been known since the time of Lavoisier that a low environmental temperature increases the metabolism in man, there are not at present concordant opinions in respect to the various aspects of this increase. In two cases out of three Lusk (1) found that one of the characteristic effects of cold was a relative increase in carbohydrate combustion. Rubner (2) also observed an increase in the respiratory quotient as well as in oxygen consumption as a result of a cold bath. Loewy (3), however, concluded as a result of an extensive study that the changes in respiratory quotient resulting from exposure to cold were due entirely to under- and over-ventilation.

This paper reports the findings pertaining to the foodstuffs oxidized during exposure to cold.

## EXPERIMENTAL

Twenty-one subjects, men and women, were exposed in a basal condition to an environmental temperature of about 2° C. and 80 per cent relative humidity. Most of the subjects were medical students and all were in normal health with the possible exception of A.D. who had been previously treated for exophthalmic goitre. Thirty-seven basal metabolism measurements are reported along with 112 observations taken during exposure to the cold environment. Two separate tests on different days were made with 15 of the subjects.

The metabolism was measured by the method of Tissot (4), essentially as modified and described by Boothby and Sandiford (5). The basal metab-

\* Submitted in partial fulfillment of the requirements for the degree Doctor of Philosophy, Department of Vital Economics, University of Rochester, Rochester, N. Y.

† On leave of absence from the Institute of Animal Nutrition, Pennsylvania State College.

olism was determined at room temperature (23–25° C.), all conditions pertaining to the proper measure of basal metabolism as outlined by Lusk (6) being rigidly observed.

An army cot, the canvas of which had been replaced by wire netting, was used throughout. This proved to be perfectly comfortable to the subject and as neither blanket nor mattress was used, this arrangement

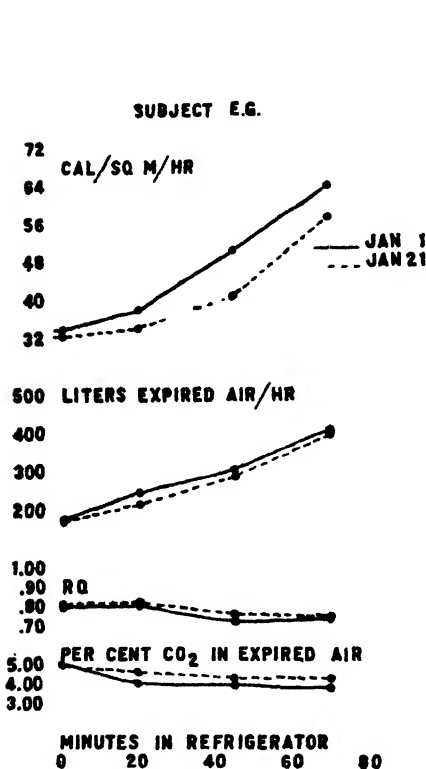


CHART 1

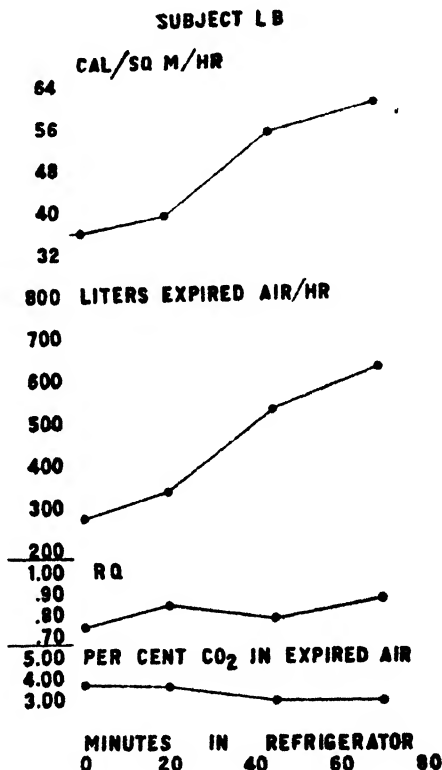


CHART 2

served to expose the whole body surface to the surrounding air. The clothing worn by all the subjects at all times was as nearly identical as possible, consisting in the case of the male subjects of shoes and stockings, light underwear, trousers, shirt, vest, and service coat. The female subjects were likewise clothed in the ordinary clothing worn in the laboratory. Though there undoubtedly may have been a slight difference in the protection offered by the clothing of different individuals, each individual wore exactly the same clothing in duplicate tests.

After the determination of the basal metabolism, the subject was moved into the refrigerator for a period of 75 minutes. No blanket, mattress, nor extra clothing was used. During the 75-minute refrigerator period, three ten-minute samples of expired air were collected for analysis.<sup>1</sup> These represented the metabolism after exposure to the cold for 20, 45, and 70 minutes respectively. An additional period was run with two of the subjects, making the total time spent in the refrigerator 100 minutes. The subjects inhaled outdoor air in all of the refrigerator periods and in most of the basal metabolism determinations, though in some cases room air was used. As pointed out by Boothby and Sandiford (5), this has no appreciable effect upon the results. The subject was connected to the apparatus by means of a mouthpiece throughout the entire refrigerator period and immediately after the collection periods was allowed to change position slightly to avoid becoming cramped.

#### *Technical Error*

Analysis of the expired air was made with a Haldane apparatus as modified by du Vigneaud (7). In the use of this excellent type of apparatus Carpenter (8) prescribes an allowable variation between duplicate determinations of .003 per cent CO<sub>2</sub> and .005 per cent oxygen. Though the agreement between duplicates in this work was not always quite so close as this, the differences were never so great as .010 per cent and usually considerably less, especially in the case of CO<sub>2</sub>. Even if the CO<sub>2</sub> and oxygen determinations are in error by .01 per cent and in such a direction as to be additive in their effects on the respiratory quotient (which as explained by Carpenter (9) is not likely to be the case) the respiratory quotient would be changed by only .003. In so far as the air analysis is concerned then, the respiratory quotient cannot be in error by more than .003. It should be borne in mind, of course, that this is true only for the analysis of expired air. In respiration chambers where the composition of the outcoming air is more nearly like atmospheric air, the accuracy of the determination of the respiratory quotient decreases accordingly. Analyses of outdoor air at intervals verified the correctness of results.

Other small errors involved with the measurement of the temperature and the volume of the expired air which have been well discussed by Boothby and Sandiford (5) cannot, even if additive, amount to 1 per cent in the computed heat production. These latter errors do not, of course, have any effect whatsoever on the respiratory quotient since it is deter-

<sup>1</sup> A few samples were taken over a somewhat shorter interval usually because of the limiting factor of the spirometer volume.

mined solely by the composition of the air. Due to the small experimental error, small differences obtained with the same or different individuals may safely be attributed to physiological variation.

This open-circuit method of indirect calorimetry presents several advantages over the closed circuit type of apparatus which have been well discussed by Carpenter (9), Murlin (10), Boothby and Sandiford (11) and Du Bois (12), and will not be further mentioned here.

Short time experiments involving a critical interpretation of the respiratory quotient must include a careful consideration of "*Auspumpung*" and the compensatory process which follows it. This important point will now be considered in connection with the results obtained.

#### *Auspumpung and the Significance of the Respiratory Quotient*

A careful consideration of the interpretation and significance of the respiratory quotient has been given by Richardson (13) and by Cathcart and Markowitz (14). They emphasize the point that the respiratory quotient represents the algebraic sum of many factors and reactions. Boothby *et al.* (15) state that the above authors in expressing caution in the literal and direct interpretation of rises in respiratory quotients that do not go above unity "have voiced the doubts of all careful workers in the subject."

*Auspumpung* is a German word which refers to the "blowing off" of more CO<sub>2</sub> than is actually produced during the time of measurement and which results from hyperventilation. A consideration of this extra CO<sub>2</sub> elimination has been well emphasized by Murlin *et al.* (16), and that it may be a complicating factor in all metabolic studies is mentioned by Colwell and Bright (17). An increase in effective respiration may be due to increase in rate or in depth or in both and may be the result of a variety of causes. Lusk (6) quotes some unpublished work by Sturgis showing the influence of voluntary deep breathing for short periods upon the respiratory quotient during which time the respiratory *rate* remained unchanged. In the first period (9 minutes) in which the subject breathed normally, the respiratory quotient was 0.80. In the second period (9 minutes) in which the breathing was much deeper though no faster, a respiratory quotient of 1.43 was obtained. During the next period, which immediately followed, and in which the subject was instructed to ignore his breathing, a respiratory quotient of 0.53 was obtained. Weiss (18) has reported essentially the same results, obtaining a respiratory quotient of 1.34 during a very short period of forced respiration and one of 0.66 in the following recovery period. An important point is the very short time necessary to eliminate a relatively large amount of CO<sub>2</sub> and this point is significant in the evalua-

tion of the few high respiratory quotients observed in this work during a few refrigerator periods. According to Bornstein and Gartzen (19) over-ventilation by voluntary effort in human subjects is limited to 50 minutes as regards the elimination of extra  $\text{CO}_2$ . By voluntary fast breathing, Coleman and Du Bois (20) obtained a change in the apparent respiratory quotient from 0.77 to 1.10 in 15 minutes. Kilborn (21), using artificial respiration in his work on decerebrate cats, discounts the high respiratory quotients obtained largely because of the washing out of  $\text{CO}_2$  from the blood.

In discussing this blowing off of  $\text{CO}_2$  Du Bois (12) says that, "Next to leaky apparatus, it has caused more trouble than any other factor. It has filled the literature with erroneous data and faulty conclusions most difficult to eradicate"—also that it "has played havoc with countless experiments on the basal metabolism and will continue to do so for years to come." In appreciation of the truth of the remarks of Du Bois, the results of this work in this respect will be fully considered and discussed.

Unfamiliarity with the apparatus may cause the subject to breathe abnormally. But that this was not the case in the results here reported is indicated by the fact that the respiratory quotient of the basal metabolism, obtained before the subject was exposed to cold, is in no case questionably high. Furthermore, most of the subjects had served as subjects before. It will be noted also that in most cases the rise in respiratory quotient occurred toward the end of the refrigerator period, after the subject had been connected to the apparatus for a considerable time.

With but very few exceptions the percentage of  $\text{CO}_2$  in the expired air decreased in successive refrigerator periods in a perfectly regular manner, indicating a continuous and slightly increasing hyperventilation. In as much as the respiratory quotient, as measured, often remained constant, in spite of some degree of hyperventilation, it seems quite possible that the true respiratory quotient tends to drop during exposure to cold. Since only 3 ten-minute air samples were taken during the 75-minute exposure to the cold, the extra  $\text{CO}_2$  eliminated as a result of hyperventilation was quantitatively measured only in so far as the extra elimination occurred during the time the samples were being taken. If it occurred *only before* a sample was taken, so that a new and lower alveolar  $\text{CO}_2$  tension had been established which was then maintained throughout the collection period, then the evidence that *Auspumpung* had taken place would consist of the observation of a large volume of expired air in proportion to the oxygen consumed, and a decreased percentage of  $\text{CO}_2$  in the expired air. The respiratory quotient, if no change was taking place as to foodstuffs oxidized,



would remain unchanged. As pointed out by Starling (22), the composition of the expired air represents the composition of the alveolar air diluted with an appreciable amount of air which remains in the "dead space." Bronchial constriction, as a reflex effect of cold, might lessen this space and thus affect the composition of the expired air. Such constriction, however, would tend to raise the percentage of  $\text{CO}_2$  in the expired air whereas in all cases the percentage of  $\text{CO}_2$  decreased and the decrease was accompanied by a large increase in ventilation.

TABLE I  
AGE, WEIGHT, HEIGHT, BODY SURFACE, AND SEX OF SUBJECTS

Subject	Sex	Age	Weight kg.	Height cm.	Surface area* sq. m.
A.A.	Female	21	64.8	166.0	1.72
E.G.	"	25	60.9	163.0	1.65
A.B.M.	"	33	58.4	164.0	1.63
M.M.	"	40	41.9	143.0	1.28
R.E.S.	Male	25	65.2	176.5	1.80
S.P.	"	22	73.8	184.0	1.96
W.D.	"	23	75.2	179.5	1.94
A.P.	"	26	69.3	171.5	1.81
J.M.	"	57	93.8	184.2	2.17
J.R.	"	15	73.6	174.0	1.88
A.D.	Female	21	51.4	164.0	1.55
E.H.	"	31	59.0	157.5	1.59
L.B.	Male	25	72.0	182.0	1.92
V.S.	"	23	75.2	179.5	1.94
T.S.	"	22	69.5	171.0	1.81
C.H.	"	27	113.4	179.0	2.28
R.D.	"	26	88.5	177.5	2.06
E.L.G.	"	25	89.3	178.0	2.09
L.M.	"	24	82.0	179.0	2.01
H.H.	"	25	78.9	168.5	1.89
E.B.	Female	26	51.4	151.0	1.46

\* Du Bois height-weight formula.

When there is no change in the oxygen consumption, the process of eliminating extra  $\text{CO}_2$  from the blood by hyperventilation must diminish the alveolar  $\text{CO}_2$  tension and, therefore, the percentage of  $\text{CO}_2$  in the expired air. Hence, any change in the  $\text{CO}_2$  content of the expired air is an indication of the  $\text{CO}_2$  changes in the blood (Finney *et al.* (23)). The volume of expired air during hyperventilation is large as compared with the oxygen consumption and a knowledge of the volume of the expired air furnished

TABLE II  
METABOLISM OF SUBJECTS EXPOSED TO A LOW ENVIRONMENTAL TEMPERATURE

Subject	Period	Refrigerator		Liters expired air per hr.	R.Q.	% CO <sub>2</sub> in expired air	Rectal temp. after basal and after exposure to cold. °C
		Temp. °C	% relative humidity				
A.A.*	Basal			232.2	.805	4.940	37.0
	1	1.3	81	377.6	.801	5.018	
	2			562.1	.827	4.760	
E.G.	3	3.3	68	574.6	.810	4.530	36.8
	Basal			191.5	.825	5.216	—
	1	1.1	81	258.5	.831	4.316	
E.G.	2			322.5	.750	4.239	
	3	5.0	58	423.5	.762	4.119	—
	Basal			185.4	.837	5.177	36.80
A.B.M.‡	1	0.6	80	232.8	.849	4.880	
	2			302.7	.790	4.605	
	3	1.9	72	422.1	.783	4.645	36.50
M.M.*	Basal			259.7	.816	4.260	37.05
	1	-0.6	78	324.2	.810	4.175	
	2			375.3	.794	3.985	
R.E.S.*	3	2.6	79	451.7	.790	4.012	37.00
	Basal			144.3	.819	4.979	36.85
	1	-0.6	84	181.6	.815	4.842	
S.P.	2			245.7	.803	4.642	
	3	1.8	79	353.7	.802	4.329	36.70
	Basal			236.4	.811	4.581	36.60
W.D.*	1	0.7	80	279.6	.836	4.730	
	2			435.2	.822	4.342	
	3	4.4	71	462.9	.793	4.507	36.40
A.P.*	Basal			285.5	.792	4.714	36.20
	1	0.6	80	326.2	.776	4.548	
	2			350.3	.778	4.391	
J.M.*	3	2.8	74	410.7	.758	4.430	36.05
	Basal			289.7	.787	4.502	36.65
	1	-0.2	84	313.3	.812	4.563	
A.P.*	2			347.1	.835	4.343	
	3	3.6	72	626.4	.872	3.829	36.40
	Basal			256.7	.854	4.558	36.55
J.M.*	1	-0.3	79	357.7	.863	4.272	
	2			427.6	.838	4.185	
	3	4.2	72	514.9	.810	4.377	36.50
J.M.*	Basal			299.6	.829	4.134	36.95
	1	0.4	79	484.2	.902	3.583	
	2			625.0	.869	3.320	
	3	3.7	71	764.0	.873	3.010	36.70

\* Average of two tests on different days.

‡ Average of 3 tests.

TABLE II (cont'd)

Subject	Period	Refrigerator		Liters expired air per hr.	R.Q.	% CO <sub>2</sub> in expired air	Rectal temp. after basal and after exposure to cold. °C
		Temp. °C	% relative humidity				
J.R.*	Basal			332.2	.801	4.342	36.70
	1	0.2	80	387.4	.802	4.310	
	2			477.8	.822	4.021	
	3	3.8	67	771.0	.870	3.534	36.50
A.D.*	Basal			249.6	.788	4.260	36.65
	1	-0.2	81	370.8	.773	4.122	
	2			411.1	.764	4.129	
	3	1.5	79	507.7	.771	4.098	36.75
E.H.*	Basal			198.4	.785	4.518	36.45
	1	0.6	80	354.9	.790	4.404	
	2			383.4	.794	4.167	
	3	3.2	75	377.7	.792	4.032	36.35
L.B.	Basal			291.6	.792	4.018	36.85
	1	1.2	81	351.3	.868	3.961	
	2			543.2	.809	3.367	
	3	4.4	63	640.5	.897	3.389	36.70
V.S.	Basal			244.7	.843	4.561	36.70
	1	-1.0	76	568.0	.879	3.783	
	2			350.2	.819	4.401	
	3	1.9	81	483.8	.800	4.527	36.70
T.S.	Basal			225.7	.816	4.776	36.70
	1	0.1	79	264.8	.860	4.507	
	2			448.1	.988	4.160	
	3	2.8	75	818.9	1.027	3.474	36.55
C.H.	Basal			354.0	.789	4.147	36.60
	1	0.0	79	362.2	.822	4.189	
	2			339.1	.790	4.114	
	3	3.1	87	363.3	.788	3.965	36.90
R.D.*	Basal			288.1	.837	4.735	36.95
	1	0.8	81	306.8	.879	4.688	
	2			282.2	.799	4.644	
	3	4.6	69	264.8	.750	4.774	36.95
E.L.G.*	Basal			256.5	.766	4.826	36.80
	1	-0.1	79	265.7	.796	4.764	
	2			266.8	.784	4.742	
	3	2.8	80	294.1	.797	4.531	36.75
L.M.*	Basal			276.8	.819	4.517	36.80
	1	0.4	80	300.7	.838	4.506	
	2			294.7	.805	4.424	
	3	3.8	71	323.6	.782	4.544	36.50
H.H.*	Basal			211.1	.823	4.932	36.65
	1	0.7	81	268.5	.900	4.584	
	2			288.1	.858	4.487	
	3	3.5	69	351.4	.823	4.382	36.40
E.B.	Basal			163.0	.752	5.006	36.55
	1	0.6	80	225.0	.785	4.732	
	2			223.9	.800	4.728	
	3	2.6	78	309.6	.777	4.680	36.05

\* Average of two tests on different days.

another criterion in evaluating respiratory quotients in metabolism tests where *Auspumpung* was suspected. Hyperventilation also results in a higher percentage of oxygen in the expired air. Observations on the rate and minute volume of respiration are directly indicative. When positive findings are made in regard to all the above factors, together with a questionably high respiratory quotient, it is safe to assume that the rise in respiratory quotient is merely superficial.

Lack of space prohibits the presentation of all the individual data which are outlined in Table II. In most cases the respiratory quotients obtained during exposure to cold were scarcely different from that of the basal. This constancy was by no means confined to those subjects who showed no marked increase in metabolism. It is true also in many cases which show a very pronounced increase in heat production and in the case of A.A., who showed the greatest increase of any subject (150 per cent), the respiratory quotient was constant indeed. Shivering by this subject was very vigorous and practically continuous. A pneumograph was not in use at the time of these tests but with only one other case was shivering so marked as to cause the spirometer bell to quiver with each expiration of the subject. Thus shivering does not necessarily produce *Auspumpung* but may be accompanied by it. The CO<sub>2</sub> content of the expired air decreased progressively with this subject as with others but no marked drop is observable between any two consecutive periods.

As mentioned above, the results of hyperventilation may be manifest in a very few minutes. An example of this is found in the case of the first test with E.G. (Chart 1). The percentage of CO<sub>2</sub> in the expired air during the basal was 5.216 while during the first refrigerator period it was only 4.316 per cent, this latter value decreasing but slightly during the subsequent refrigerator period. The first air sample in all cases was collected during a 10-minute interval which began 15 minutes after the subject entered the refrigerator. In this instance considerable *Auspumpung* apparently occurred during the 15 minutes *before* the sample was taken, as indicated by the pronounced and sustained drop in the percentage of CO<sub>2</sub> which is not accompanied by an increase in the respiratory quotient. All the observations pertaining to *Auspumpung* mentioned above indicate that in the second test with this subject the same thing occurred to a less degree and somewhat later, so that the blowing off of CO<sub>2</sub> was still taking place to some extent during the first collection period. This is indicated by the rise in respiratory quotient, the drop in the percentage of CO<sub>2</sub> in the expired air, and in an increase of 25.5 per cent in the volume of expired air over the basal, with no increase in oxygen consumption. The change in re-

spiratory quotient from 0.837 to 0.849, though appreciable, certainly does not indicate increased combustion of carbohydrate. An exhaustive consideration of all instances showing a rise in respiratory quotient leads to the inevitable conclusion that the rise results from the blowing off of  $\text{CO}_2$ .

As a further check on the superficiality of the few high respiratory quotients obtained, a "recovery period" was run on subject L.B. (Chart 2). The apparent respiratory quotient which was 0.897 during the last refrigerator period dropped to 0.730. This 10-minute period at room temperature, was taken 20 to 30 minutes after exit from the cold. A sample taken sooner would undoubtedly have given an even greater drop in the respiratory quotient.

The respiratory quotients obtained with subject V.S. are in keeping with the very exceptional values obtained as to the irregularity of the oxygen absorption, ventilation, and  $\text{CO}_2$  content of the expired air.

Subject T.S. showed a progressive hyperventilation throughout and the apparent respiratory quotient rose to more than unity in the last refrigerator period. A regular decrease in the percentage of  $\text{CO}_2$  in the expired air with an increased respiratory volume greatly in excess of the increased oxygen absorption is apparent in these periods.

An inspection of data obtained following the ingestion of 300 grams of meat, a preliminary report of which is given elsewhere (24), shows that the percentage increase in volume of expired air never exceeds the percentage increase in oxygen consumption. Exposure to cold, on the other hand, especially in periods where a rise in respiratory quotient is observed, results in a very much larger increase in expired air volume than in oxygen absorption, and the  $\text{CO}_2$  content of the expired air invariably decreases. The same apparatus was used in both cases.

A special experiment was performed with subject T.S. By use of two spirometers all of the expired air was collected over the entire observation period of 1 hour and 34 minutes. The details of the experiment were as follows: The two spirometers were swept out with expired air from the subject who, in a basal condition, was lying on a cot in a room at  $23^\circ \text{C}$ . By throwing the valve on one of the spirometers the air was deflected from one into the other. After a 12-minute collection period, and as soon as the valve had been turned, the room was cooled by opening windows. A sample was taken from one spirometer while the other was being filled. After the sample was taken, the spirometer was completely emptied to be ready for the next interval. There was of course a slight contamination from the small amount of air left in the spirometer when it became mixed with the sample next collected in it. The residual air was very small in amount,

however, and the very small error involved tends primarily to make the example of *Auspumpung* less pronounced rather than to exaggerate the situation. The following table shows beyond any doubt that this subject reacted in this experiment as he did in the refrigerator and that the pronounced rise in respiratory quotient is entirely superficial.

DATA OBTAINED DURING EXPOSURE TO COLD AND DURING THE RECOVERY PERIOD

Time in minutes		R.Q.	Liters expired air per hour		% CO <sub>2</sub> in expired air
			Total	Per liter O <sub>2</sub> absorbed	
Basal	12.5	.841	221.5	16.7	5.072
Exposed to cold	11.5	.863	263.9	16.8	5.185
	16.3	.861	224.5	16.9	5.144
	8.4*	1.003	343.1	20.9	4.835
	7.2	.749	295.0	15.3	4.939
	7.7	.841	237.0	16.7	5.080
	12.1	.794	200.8	15.0	5.329
	10.3	.807	229.2	15.1	5.381
	8.0	.800	203.3	14.9	5.418
Average		.840			

\* Vigorous shivering.

Vigorous shivering occurred during the third period following the basal. At the end of this period the windows were closed and the subject covered with blankets. The room temperature had dropped to about 10° C. with rapid movement of air currents. If the subject could have been warmed up more rapidly, a greater drop in respiratory quotient would have been apparent as well as a quicker return to the basal value.

The demonstration is so clean cut as to necessitate but little comment. The basal respiratory quotient of 0.841 is to be compared with the average of all the periods which is 0.840. The "weighted" or true average of all happens to be exactly 0.841. Exposure to cold accompanied by vigorous shivering, therefore, has no effect whatsoever on the true respiratory quotient.

A noteworthy observation is that the percentage of CO<sub>2</sub> during the period giving a respiratory quotient of 1.003 dropped only to 4.835 per cent from 5.144 per cent—its value in the preceding period. The short period of 8 minutes was thus long enough to give a high respiratory quotient but too short to lower the alveolar CO<sub>2</sub> tension markedly. A fact of significance is that the volume of air expired per liter of oxygen absorbed is greatest where the respiratory quotient is highest. Thus the instances

which show a definite rise in respiratory quotient after exposure to cold have all the earmarks of extra  $\text{CO}_2$  elimination.

### SUMMARY

Thirty-seven basal metabolism measurements on humans are reported along with 112 observations taken during exposure to cold. A critical study of the apparent respiratory quotients obtained, clearly indicates that exposure to cold does not favor any preferential oxidation of carbohydrate. Depletion of the glycogen stores is, therefore, merely associated with, and proportional to, the increased total metabolism.

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Unselfish coöperation by the voluntary subjects and kindly advice by Prof. J. R. Murlin are gratefully acknowledged.

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## THE EFFECTS OF LOW ENVIRONMENTAL TEMPERATURE UPON METABOLISM\*

### II. THE INFLUENCE OF SHIVERING, SUBCUTANEOUS FAT, AND SKIN TEMPERATURE ON HEAT PRODUCTION

By RAYMOND W. SWIFT

(From the Department of Vital Economics, University of Rochester, Rochester, N. Y.)

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IN MEASURING the metabolism of a fasting man exposed to cold, Voit (1) found an increase of 34.5 per cent in the  $\text{CO}_2$  production which occurred in the absence of shivering. The work of Rubner and Lewaschew (2) supported Voit's observations. Campbell *et al.* (3) determined the metabolism of subjects exposed to the rather cold open air and concluded that shivering was not necessary to raise the metabolism. Hill and Campbell (4) in similar experiments found increases of about 43 per cent during the exposure of normal and pathological subjects to cold outdoor air. The work of Cannon *et al.* (5) with humans indicates that a considerable increase in metabolism in response to a "heat debt" may ensue without any accompanying shivering.

Loewy (6), however, concluded that increases in metabolism took place only when accompanied by shivering. This latter opinion was also expressed by Johansson (7) and by Sjöström (8) both of whom measured the  $\text{CO}_2$  output when the subject was naked and exposed to cold. Benedict (9) is inclined to agree with them, and Morgulis (10) concludes, from results obtained with dogs, that metabolism increases on exposure to cold only because of a heightened state of muscle tension.

The primary purposes of the work reported in this paper are: First, the measurement of the increased metabolism; second, the determination of whether or not shivering is necessary for this increase; and third, the relation of skin and body temperature to the onset of shivering.

#### EXPERIMENTAL

The experimental procedure involving the metabolic measurements on 21 human subjects has been described in the preceding paper. In addition to the respiratory quotient and the heat production, which were computed from the air analysis, the nitrogen elimination was measured in several

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cases. Blood sugar was determined on samples taken just prior to and immediately following the exposure to cold. In some of the early experiments the operator entered the refrigerator immediately after each 10-minute collection period for a time just long enough to determine the pulse rate. This was discontinued later, however, as the importance of the data on this point (since the pulse was always found to be the same or slightly

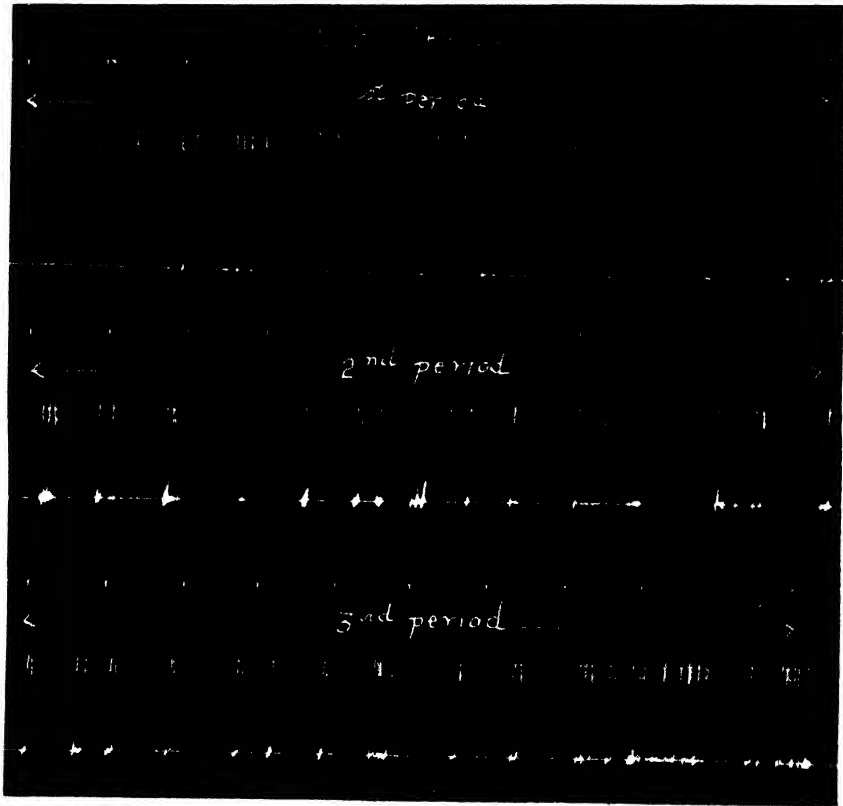


FIG. 1

slower than during the basal) seemed hardly to justify the disturbance caused by entering the refrigerator. Subsequently the pulse was taken immediately after the refrigerator period had ended and while the subject still lay on the cot. Rectal temperatures by clinical thermometer were taken immediately following the basal determination and just after the exposure to cold. The temperature and humidity of the refrigerator were measured by means of a hygrometer.<sup>1</sup>

<sup>1</sup> "Hygrodeik," made by Taylor Instrument Co., Rochester.

Since shivering was one of the important factors in this study, a special effort was made to record it. The six legs of the cot rested on automobile valve springs and a pneumograph was connected to the side of the cot and to the refrigerator wall. The entire pneumograph system was kept under pressure ( $\frac{1}{4}$  inch water) to insure sensitivity.

In the earlier trials before the pneumograph was used, a record of shivering was obtained by use of a signal magnet. The subject held a small con-

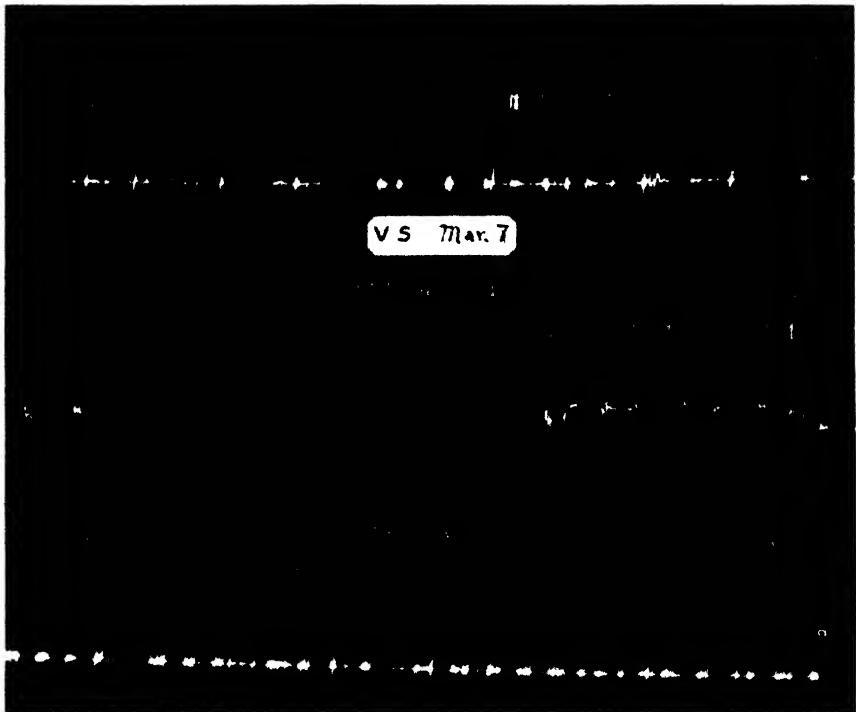


FIG. 2

tact switch which was pressed and held in contact during each transitory period of shivering. To confirm the pneumograph record and to distinguish between shivering and any voluntary movement, the use of the signal magnet was continued throughout. The record of the pneumograph is written directly under the one made at the same time by the signal magnet (Figs. 1 and 2). The top line in all cases indicates the time in minutes. When the switch was held in contact the signal magnet needle was in the "up" position as seen in the photographs. The excellent parallelism between the two records is apparent in the records of shivering.

A sensation of "tightening up" or of increased muscular tonus before actual shivering began was described by the subjects. Some found it difficult to decide whether the chills which "ran up the back" constituted shivering, so that in many of the refrigerator periods there is, therefore, a record of initial shivering as indicated by the signal magnet which is not substantiated by the pneumograph record. The duration of the refrigerator periods was one hour and fifteen minutes.

The onset of shivering under the conditions of the experiment was so gradual as to make impossible any definite record of its actual beginning. The relative impossibility of lying in a relaxed manner in a cold environment has to be experienced in order to be appreciated. The increased "tension all over" experienced by the subjects has no quantitative measurement in this work but cannot be ignored as a source of increased metabolism. Furthermore, very slight tremors in localized parts of the body, too slight to be recorded by the pneumograph, preceded general shivering. After a certain time of exposure it is impossible to avoid vigorous shivering in the leg muscles when the knees are drawn up, even at a time when the subject, lying flat, might well record "no shivering." Shivering often began by very slight and rapid twitches of various muscles in the back and shoulders. There is, therefore, no sharp line of demarcation as to exactly when shivering begins. The signal magnet record is of value in indicating its onset as judged by the subject. Certainly the first slight but definite twitching of the muscles would not be visible to an observer. A translation of a description of the onset of shivering given by Fredericq (11) many years ago is pertinent:

When the body is exposed to cold, one feels a certain degree of rigidity in all the muscles of the body, especially in the arms and legs. I have thoroughly verified this rigidity in experiments in which I stayed in the cold without clothing; I have been able to ascertain that it is intimately related to the involuntary trembling, which follows reflexly, when the action of the cold is extended further. The tension increases and ends by transformation into intermittent trembling.

The criterion of effective exposure or "the tendency to shiver" which Barcroft and Marshall (12) judged by a tendency of the knees of the sitting subject to come together would indicate that there is an increase of muscular tonus (and of oxygen absorption) which precedes visible shivering. Although the tendency to shiver in their experiment was not noticeable to an observer, the increased tonus may have been responsible for the increased metabolism. This factor is no doubt concerned in those cases of Loewy in which the metabolism increased without noticeable shivering.

No ill effects of any sort were experienced by any of the subjects following the exposure to cold.

*Heat Production*

The heat production was calculated by use of the Zuntz and Schumberg tables for the calorific value of oxygen, without allowance for the protein metabolism. The error involved is small indeed and, as pointed out by Boothby and Sandiford (13), the method is probably just as accurate for short time experiments on account of the lag in the nitrogen elimination. The calorific value of oxygen per liter was used according to the respiratory quotient obtained. This procedure undoubtedly gives results somewhat too high in those cases in which the superficial high respiratory quotient has involved the use of a higher calorific value. However, it has seemed the only consistent procedure possible. The heat production is given in Table I and in the charts and only a few comments are necessary.

Comparison of 33 basal metabolic rates of adults as obtained in this work with three standards shows that the majority of the results are lower than the prediction value.

	Average deviation in per cent		
	Du Bois formula	Harris-Benedict formula	Dreyer formula
Regardless of sign . . . . .	.8.4	8.6	6.9
Plus . . . . .	.6.2	10.0	10.3
Minus . . . . .	.8.9	8.2	5.8

According to the Du Bois and the Harris-Benedict standards, 26 cases were below the prediction value. The Dreyer formula showed 25 were below. The two trials with subject A.D. have been omitted in the averages as this subject was a case of moderate hyperthyroidism. The basal metabolism of this subject was high to the extent of 11.9 per cent, 13.6 per cent, and 14.6 per cent, according to the prediction value of Du Bois, Harris-Benedict, and Dreyer respectively. Subject A.B.M., though presumably a normal subject, had a high basal rate which in three separate tests averaged considerably above the standards. This average deviation was 9.7 per cent, 14.0 per cent, 15.4 per cent, according to the formulas of Du Bois, Harris-Benedict, and Dreyer respectively.

It is perhaps not surprising that most of the values were found to be lower than the standards. Krogh (14), MacLeod and Rose (15), Hafkesbring and Borgstrom (16), and Du Bois (17) have reported findings which indicate that the standards are too high when the modern methods of rest and quiet are observed.

The parallelism between the heat production and the volume of air breathed is so evident in the charts as to need no particular comment. Though the heat production can be computed quite accurately from the

TABLE I  
HEAT PRODUCTION, NITROGEN ELIMINATION, AND BLOOD SUGAR OF SUBJECTS EXPOSED TO COLD

Subject	Heat production per square meter per hr.				Nitrogen elimination per hr.		Blood Sugar	
	Basal	Period 1	Period 2	Period 3	Basal	During exposure to cold	Basal	After exposure to cold
	Cals.	Cals.	Cals.	Cals.	Grams	Grams	Mg. per 100 cc.	Mg. per 100 cc.
A.A.	38.74	58.11	82.67	96.37				
A.A.	40.08	72.17	96.95	82.09				
E.G.*	34.41	36.27	47.07	62.09	.70	.55	91	97
A.E.M.†	39.70	48.69	55.27	66.73	.54	.50		
M.M.*	32.69	40.49	52.91	63.75	.34	.35		
R.E.S.*	35.41	42.14	58.60	69.43			67	65
S.P.	41.19	46.10	47.73	57.59			75	75
W.D.*	40.22	43.27	44.59	69.46			70	71
A.P.*	36.50	46.79	56.67	73.15			85**	76**
J.M.	33.00	42.62	52.68	58.14	.53	.52		
J.R.*	45.55	52.75	58.94	80.37	.48	.65		
A.D.*	41.28	60.15	67.44	82.15	.51	.68	75	77
E.H.*	34.01	62.20	60.07	57.37				
L.B.	37.32	40.37	55.94	61.30				
V.S.	33.34	62.06	47.13	68.17				
T.S.	34.88	37.06	51.93	76.38				
C.H.	38.69	38.71	36.75	38.00			80	71
R.D.*	37.93	38.56	37.85	38.46			86	72
E.L.G.*	36.58	37.01	36.68	38.15		.74	81**	80**
L.M.*	36.33	38.67	38.37	44.34	.51			
H.H.*	35.47	35.34	38.50	47.69			63	60
E.B.	34.94	44.05	43.18	60.40			80	77

\* Average heat production of two trials.

\*\* Average of two trials.

† Average heat production of three trials.

volume of expired air in many cases, such a procedure is not very trustworthy, especially in cases of hyperventilation.

As a general rule shivering increased steadily in vigor and in frequency as the period of exposure continued. Lack of space prohibits the presentation of all the records of shivering which show very clearly the dependence of increased metabolism upon the duration and vigor of shivering.

In order to obtain a roughly quantitative value of the increase in heat production caused by shivering, some actual measurements of these intervals of time, as indicated by the signal magnet, were taken. The periods used are indicated in the following table. The record made by the signal

TABLE II  
RELATION OF TIME SPENT IN SHIVERING TO THE INCREASE IN METABOLISM

Subject	Date	Heat Production, increase over basal	Time spent in shivering*	Increase in heat production caused by shivering 1% of the time
		Per cent	Per cent	Per cent
A.P.	Feb. 12	68.5	20.8	3.3
" "	Mar. 14	130.8	48.8	2.7
H.H.	Mar. 3	6.2	5.9	1.1**
" "	Mar. 12	63.4	17.1	3.7
L.B.	Feb. 26	64.3	18.5	3.5
R.E.S.	Feb. 20	109.4	18.4	5.9
" " "	Mar. 20	83.4	14.3	5.8
A.D.	Feb. 15	116.5	15.0	7.8
V.S.	Mar. 7	104.5	43.7	2.4
Average				4.0

\* Third refrigerator period.

\*\* Shivering not so marked in this test as in the one of March 12.

magnet in these periods is not complicated by any compromise of judgment by the subjects as to whether shivering occurred, for shivering had begun considerably earlier in every case and the record of the signal magnet is perfectly confirmed by the pneumograph record. However, since the accuracy of the signal magnet record depends on the attention of the subject, the pneumograph record is given the more consideration in computing these intervals of time.

In so far as this method of getting at the quantitative effect of shivering on metabolism tacitly assumes the metabolic rate between successive intervals of shivering to be at the basal level, it is of course inaccurate. The variability in the extent of shivering has been brought out early in the dis-



cussion and it is to be understood, of course, that no strict quantitative measure of shivering in these experiments is possible because intermittent shivering, though vigorous, definite, and well-defined in duplicate experiments, is not necessarily identical in its extent or effects. Du Bois (17) imitated as best he could the shivering of a malarial chill and found about the same increase in metabolism (170 per cent) as was observed in that of the patient.

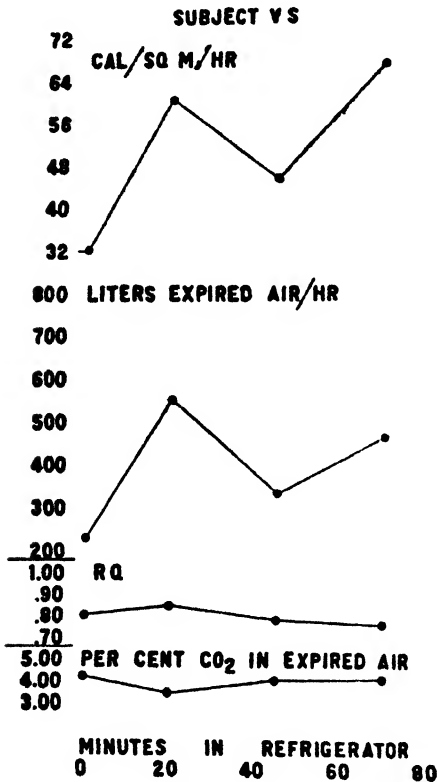


CHART 1

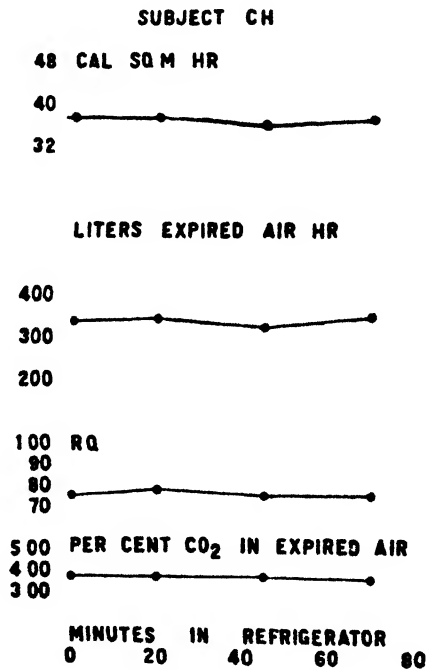


CHART 2

The exceptional behavior of subject V.S. (Chart 1), showing a drop in heat production in the second refrigerator period, is associated with the fact that shivering, which had been very pronounced during the previous half hour, entirely stopped for 6 consecutive minutes, which time constituted 60 per cent of the collection period of the air sample. The subject reported that he did not "feel cold" during this particular interval. The results obtained with this subject are exceptional, as regards the lull in shivering, but quite characteristic as regards the relation between shivering and heat production.

Subject A.A. reached the same peak of heat production in both tests but sooner in the second than in the first. The greater drop in body temperature during the second trial would account for the difference obtained in the final periods of these two days, since an actual cooling off of the body would not be measured by indirect calorimetry. In fact Barr and Du Bois (18) show that a body temperature change during a calorimeter test may be measured by the discrepancy between direct and indirect calorimetry. In so far as a slight drop in body temperature occurred in practically all cases, the above observation applies to all the measurements of heat production. Subject A.A. was in unusually good physical condition which may be related to the great height of heat production attained. Benedict and Smith (19) have shown that athletes have a higher metabolism than non-athletes. Du Bois (17) likewise attaches importance to differences in athletic training. Lusk (20) found the most pronounced reaction to a cold bath with a subject who was a well-trained athlete. Kaup and Grosse (21) report several definite effects as a result of two months' training. These changes were a decrease in body weight and chest circumference along with an increase in chest expansion and vital capacity. Training lowered the blood pressure and increased the circulatory minute volume because of the increased systolic discharge of the heart. The rise of alveolar  $\text{CO}_2$  tension was less marked in training. The respiratory quotient both at rest and during exercise was increased by training. The trained subject showed a much quicker return to normal after exercise in respect to heart rate, blood pressure, and oxygen consumption. The efficiency with which exercise was performed was increased.

The temperature and humidity of the refrigerator were not appreciably different in duplicate tests. The heat eliminated by the subject warmed the refrigerator somewhat and, since relatively little water is eliminated through the skin under the experimental conditions, the humidity usually fell.

Variation in duplicate tests is apparently the result of some physiological difference too slight to be detected by comparing duplicate basal rates but which is manifested on exposure to cold. The subjects who showed little or no change in heat production will be considered when subcutaneous fat is discussed.

### *Respiratory and Pulse Rates*

A complete record of respiratory and pulse rates shows in the majority of cases that both decreased slightly during the exposure to cold. This decrease, it is of interest to note, took place while the metabolism was rapidly

increasing. The work of Barcroft and Marshall and of Loewy, above referred to, showed likewise a decrease of the pulse rate and respiratory rate respectively. The failure of the pulse rate to increase does not exclude the possibility of increased secretion of epinephrin since the nervous system of the subject was of course intact. In no case, except J.M., January 19, did hyperventilation involve a faster respiratory rate. Harris and Benedict (22) report a poor correlation between pulse rate and total (basal) metabolism. Murlin and Greer (23) found that a cold bath increased the pulse volume of the dog. Marshall (24) produced marked shivering in a dog by means of a cold bath and obtained only a slight increase in heart rate but a marked increase in output per beat.

### *Subcutaneous Fat*

That subcutaneous fat may be of importance as a factor in heat regulation, seems self-evident. Benedict *et al.* (25) found bigger increases in pulse rate with fat subjects exposed to hot air than with normals. Lusk (26) considers subcutaneous fat as a blanket which delays the chemical regulation of body temperature.

Measurements of subcutaneous fat were taken as prescribed by Franzen (27). These directions call for two measurements on the biceps, four on the triceps, and four on the calf of the leg. Since these measurements were closely alike for a given locality of the body, only one value for each is recorded. In addition to these measurements, three additional ones were taken at points on a transverse line three centimeters above the navel, one directly above, one at the same level at the side, and one 5 centimeters from the middle of the back. The calipers were set at 40 in obtaining the readings as was the case in the triceps and calf measurements. The results are given in Table III. The variation in the distribution of fat and the somewhat questionable significance of the procedure make the actual figures obtained of limited value. The measurements on the body proper and those on the arm and leg are given equal consideration. Though the measurements of subcutaneous fat do not confirm in a quantitative way the reaction of the subjects to cold, it is apparent that the chief reason for the tremendous variation in response to cold of different subjects is the amount of protection afforded by the insulating fat. The differences in metabolism sometimes noted with a subject in duplicate tests can not of course be considered a function of fatness. The simple comparison of weight (kg.) per unit of height (cm.), which gives a general idea of the type of stature of the individual, shows that the particular individuals who give a value of more than 0.45 are also the individuals who show little or no in-

TABLE III  
MEASUREMENTS OF SUBCUTANEOUS FAT

Subject	Biceps	Triceps	Calf	Abdomen	Side	Back	Sum
A.A.	15	22	29	19	12	19	116
E.G.	15	25	20	22	26	29	137
A.B.M.	14	21	24	22	11	21	113
M.M.	14	24	23	20	23	21	125
R.E.S.	11	14	21	15	16	18	95
S.P.	10	14	20	15	14	16	89
W.D.	12	19	18	24	20	22	115
A.P.	11	12	17	19	16	19	94
J.R.	14	22	26	20	20	21	123
A.D.	14	23	24	21	21	22	125
E.H.	16	25	17	25	24	30	137
L.B.	11	18	16	18	20	23	106
V.S.	12	18	17	22	22	21	112
T.S.	11	16	18	15	16	21	97
C.H.	15	22	30	34	28	30	159
R.D.	15	27	16	31	33	35	157
E.L.G.	14	29	23	22	23	28	139
L.M.	11	20	23	20	23	29	126
H.H.	13	27	27	32	24	29	152
E.B.	21	20	20	18	19	25	123

crease in metabolism when exposed to cold under the experimental conditions. The only exception to this is subject J.M. Although this subject had a rather large weight per unit of height, the metabolism was markedly influenced by the cold. According to Munk (28) shivering occurs in older persons under conditions which would not cause shivering in younger ones. This being true, it would seem to explain the results obtained on this subject. One very fat subject (Chart 2) showed absolutely no increase in metabolism during exposure to cold. Ebbecke (29), from experiments on human subjects, concluded that the sensation of cold or warmth on the skin depends upon the difference in temperature in the layers of the skin immediately adjacent to the cold and warm receptors and that the temperature sense nerve endings for heat are situated in the deeper layers of the skin, while the cold sense nerve endings lie at the junction of the epidermis and the cutis vera. Inasmuch as subcutaneous fat seems to protect the cold sense nerve endings, the effective fat must lie between them and the surface.

#### *Nitrogen Elimination*

The higher nitrogen values obtained (Table I) in some of the cases might seem to indicate an increased protein katabolism during exposure to cold. However, considering the varying conditions, the periods of collection

were rather short to allow close interpretation of the results. The periods of collection varied from one hour and a quarter to two hours and a quarter. An exposure to cold has a marked diuretic effect by decreasing the loss of water through the skin, and by action through the nervous system. The diuresis would tend to give higher nitrogen values by reason of its "washing out" effect. Also, the total metabolism of subject E.L.G., whose nitrogen elimination apparently increased 45 per cent, rose only to 5.9 per cent above the basal during the last refrigerator period. Some of the other subjects whose metabolism increased very greatly show no change in nitrogen elimination.

The experiment was not planned for a special study of the protein katabolism, the above observations being included in a supplementary manner. Also, inasmuch as there was no rigid control of water intake, the results must be only broadly interpreted. The results of Voit (1), cited above, should be considered more significant than these since he collected 6-hour samples for the determination of nitrogen. Chaikoff and MacLeod (30) found no change in nitrogen elimination as measured by 24-hour samples from dogs exposed to cold. Cohn and Gessler (31) have reported similar findings. The results given in Table I are interpreted as indicating no significant change in protein metabolism during exposure to cold.

### *Blood Sugar*

The method of preparing the blood filtrate was that of Haden (32) and sugar determination was carried out as outlined by Benedict (33). No comment on the results shown in Table I is necessary except that the change in blood sugar was in general hardly outside the limit of experimental error. Freund and Marchand (34) found increases in blood sugar in the dog when exposed to cold. Kramer and Coffin (35) found that the exposure of a dog to a low environmental temperature raised the blood sugar only if the exposure continued for a day or more. Shivering did not raise the level of blood sugar. Britton (36) administered insulin to normal cats, and when the blood sugar had fallen to the convulsive level, placed them in cold water with the result that the blood sugar rose and shivering occurred. In cats without active adrenals the blood sugar changes were small and shivering never occurred. They showed no tendency to regain their previous body temperature. The injection of epinephrin raised the blood sugar and restored shivering. This shows a close relationship between epinephrin, blood sugar, shivering, and body temperature control.

Dworkin and Finney (37) produced artificial hibernation in the woodchuck by the administration of insulin. The animal, in profound hypo-

glycemia, lost its power of temperature control and in an environment even moderately cool, passed into a state of hibernation. Injected glucose terminated the condition, produced shivering, and the recovery of body temperature.

However, the nervous control of body temperature can not be dependent upon the blood sugar level, for heat regulation is intact in the phloridzinized dog in spite of the low level of the blood sugar.

### *The Role of Epinephrin in Temperature Regulation*

That epinephrin secretion can be increased by cold, and if so increased may affect heat production, seems indicated by the reports of Boothby and Sandiford (38), Hunt and Bright (39), and by Cori and Cori (40). The latter showed that epinephrin did not raise the respiratory quotient of fasting rats although the heat production increased 17.3 per cent. During glucose absorption they found the heat production of rats injected with epinephrin to be increased 16.4 per cent and that the extra heat, in the presence of a carbohydrate plethora, was furnished solely by the oxidation of fat. In experiments on human subjects (41) they found that injection of epinephrin did not raise the arterio-venous blood sugar difference though the blood sugar level invariably rose.

That epinephrin increases metabolism is well established. Evidence that it is secreted in increased amount as a result of exposure to cold is indicated by the work of Hartman *et al.* (42). Hartman and Hartman (43) also found cold to be effective, independent of excitement or muscular activity.

Cannon *et al.* (5) observed a considerable increase in the denervated heart rate of the cat, when exposed to cold, which did not take place when one adrenal was removed and the other denervated.

Demonstration as to the increased secretion of epinephrin was impossible in the experiments of this paper. It seems significant, however, that Cori and Buchwald (44) found from the intravenous injection of epinephrin into normal men that the minimum dosage of epinephrin sufficient to increase the oxygen consumption was twice as great as that necessary to produce hyperglycemia. The unchanging level of the blood sugar during exposure to cold is, therefore, strong evidence that increased secretion of epinephrin did not accompany the increases in metabolism.

### *The Role of Shivering in Body Temperature Regulation*

Since shivering is a characteristic response to cold, it may be properly considered as part of the heat-regulating mechanism.

Richet (45) distinguished between a shivering reflex caused by application of cold to the skin and that caused by a cooling of the whole body. The first does not take place with a dog under anaesthesia. The same author (46) showed that reflex shivering took place before there was any appreciable drop in body temperature. In the case of an animal under anaesthesia, however, shivering may not occur until there is a drop in body temperature of as much as 6° C. Sjöström (8) believed shivering to be a reflex initiated by the cold receptors and that skin temperature was the decisive factor for its onset.

Sherrington (47), with dogs, has shown the clearness with which spinal transection separates the body in regard to its response to cold. Dogs with spinal transection placed in ice water showed no sign of shivering behind the transection though there was acute shivering in the region forward from it. Apparently shivering can not be produced as a spinal reflex.

According to O'Conner (48), shivering in cats and rabbits depends upon the temperature of the brain rather than on that of the skin. The animals responded to brain temperature controlled by warming or cooling the carotid vessels. These animals were under anaesthesia, however, and are therefore not comparable to normal animals which shiver when no appreciable drop in body temperature takes place.

It has been shown by Finney *et al.* (49) that the shivering reflex disappears in dogs given amytal when the temperature drop is from 9 to 17° C. depending on the depth of anaesthesia and the condition of the animal. Dworkin (50) recently states that the exact significance of shivering is still not settled, "while very little indeed is known concerning its mode of origin and its nervous control."

The work of Voit indicates a chemical control of body temperature independent of shivering. This work, confirmed by Rubner and Lewaschen, constitutes the old work upon which the conception is based. The experiments were of several hours' duration in all cases and the results are without doubt perfectly trustworthy. Although this work was done before the importance of "extra CO<sub>2</sub>" elimination was fully appreciated, the experiments were fortunately of several hours' duration, which minimizes any error of this nature. It should be borne in mind, however, that the noteworthy increases in metabolism occurred at temperatures not far removed from those which produced visible shivering.

The excellent work of Cannon certainly indicates the production of epinephrin in cats when exposed to cold, or when a "heat debt" is incurred by ingestion of cold water. His experiments on men showed that a "heat debt" incurred in the same way, resulted in an increased metabolism

though no shivering occurred. He points out that the room temperature was an important factor in its effect on the extent of the increases obtained. Shivering invariably caused a marked rise in metabolism.

Shivering occurred in cats in which the adrenals were lacking much sooner and for longer intervals than in normal animals. "Thus when the heat-producing service of the adrenal medulla is lacking, the shivering mechanism is resorted to."

The inactivation of the adrenal glands, however, not only removes their "heat-producing service," but also any and all other effects which they may normally exert. Aside from any power to increase the metabolic rate, the effects of epinephrin are characterized by increases in blood sugar, constriction of peripheral blood vessels, and by increases in heart rate and blood pressure. The extent to which these known effects influence metabolic rate is not precisely known. It may also be questioned whether a "heat debt" incurred by the ingestion of cold water involves the same mechanisms as does the stimulus of cold on the skin.

Shivering induced by a cold douche or by exposure of the naked body to cold air takes place independent of any appreciable drop in body temperature. The "heat debts" in Cannon's work apparently caused a definite drop in body temperature as judged by the axilla temperature reported and certainly lowered the temperature of some of the inner organs. "Heat debt" brings about conditions which do not duplicate those caused by exposure to cold air. Shivering, when produced under the two sets of conditions, is brought about in quite dissimilar manners,—one from within and one from without. It is at least conceivable that the factors involved in maintaining a constant body temperature may be used, under the two conditions, to significantly different extents.

The experiments of Johansson and those of Sjöström also involved the determination of  $\text{CO}_2$  as a measure of the metabolism. Cannon criticizes their conclusions,—that increases in metabolism without shivering never occur—, in view of the fact that increases in  $\text{CO}_2$  output were observed, from 7 to 28 per cent, which were not accompanied by shivering. Evidently these authors as well as Loewy fully appreciated that there was a gradual increase in muscle tension which precedes shivering and which merges into it. They were willing to ascribe to it such increases in metabolism as they obtained not due to visible shivering. The onset of shivering caused by exposure to cold air has been described in detail earlier in this paper and a parallel description taken from the words of Fredericq.

All the periods in which an increased metabolism over the basal occurred without shivering have been listed in Table IV, the average increase being



11.3 per cent. Experiments in which no shivering occurred subsequently have been omitted as there is no exact way of determining how soon shivering would have taken place. Inclusion of such values would not raise the average. Differences between refrigerator periods and the basal metabolism in such cases may represent merely physiological variation and can not with certainty be ascribed to the environmental temperature. It is important to note, however, in this connection, that subject R.D.,

TABLE IV  
INCREASES IN HEAT PRODUCTION NOT ACCOMPANIED BY SHIVERING

Subject	Date	Basal Cal.	In Refrigerator	
			Cal.	Per cent increase
R.E.S.	Feb. 20	34.46	41.21	19.6
" " "	Mar. 20	36.36	43.06	18.4
S.P.	Feb. 23	41.19	46.10	11.9
" "	" "	"	47.73	15.9
W.D.	Feb. 27	39.26	42.21	7.5
" "	" "	"	42.29	7.7
" "	Mar. 13	41.18	44.32	7.6
" "	" "	"	46.88	13.8
A.P.	Feb. 12	35.67	38.82	8.8
J.M.	Jan. 19	33.59	36.52	8.7
J.R.	Jan. 7	45.43	55.57	22.3
" "	" 31	45.67	49.92	9.3
" "	" "	"	53.97	18.2
L.M.	Feb. 22	35.86	38.48	7.3
" "	Mar. 1	36.80	38.85	5.6
" "	" "	"	38.70	5.2
H.H.	Mar. 12	35.03	36.24	5.2
Average				11.3

February 11, who showed an increase of 9.0 per cent during the last refrigerator period, began to shiver just after the last air sample had been taken.

In order to determine whether increase in muscle tension could account for such increases in metabolism as are shown in Table IV, the following test was made on J.R. who had previously been a subject in many refrigerator trials. This particular experiment was done at room temperature. The basal metabolism was determined in the usual manner. Immediately afterward the subject was instructed to imitate as faithfully as possible the increase in tension which he had experienced during refrigerator periods. This increased tension has been described in some de-

tail above and in this experiment no muscular movement whatever took place. The results are shown in tabular form:

	Basal	Voluntary muscular tension	Per cent increase
Liters CO <sub>2</sub> per hr. . . . .	14.17	25.03	76.6
" O <sub>2</sub> per hr. . . . .	17.03	23.15	35.9
R.Q. . . . .	.832	1.081	
Liters expired air per hr. . . . .	338.6	766.3	126.3
Liters expired air per hour per L. O <sub>2</sub> . . . . .	19.9	33.1	
% CO <sub>2</sub> in expired air . . . . .	4.224	3.306	

Muscular tension alone, without regard to shivering or exposure to cold, may thus increase the metabolism 36 per cent. Making generous allowance for exaggeration in an honest attempt to reproduce the involuntary tension in refrigerator experiments, there is still enough margin left to account for any increases encountered which did not involve shivering. If judged by the CO<sub>2</sub> production, a much larger increase is noted. The great discrepancy between the increase in the CO<sub>2</sub> production and in the oxygen absorption shows the possibility of drawing erroneous conclusions from CO<sub>2</sub> measurements over short periods. Incidentally, the data in this test exhibit all the earmarks of *Auspumpung* described in the first paper of this series.

The important point, however, to the general subject of this paper is that by a failure to relax, the oxygen consumption may be increased 36 per cent. The relative impossibility of relaxation when exposed to cold has already been mentioned. It seems, therefore, logical to attribute the increase in metabolism in those periods listed in Table IV to increased muscular tension.

The term "no shivering" is not usable as a critical measure of condition. Whereas "shivering" designates a more or less definite condition invariably associated with increased metabolism, "no shivering" may indicate anything from complete relaxation, with no increase in metabolism, to a state of muscular tension involving a very pronounced increase in metabolism. This is a point to be stressed since the chief objection to the claims of Loewy, Johansson, and Sjöström is that definite increases in metabolism occurred with "no shivering."

Shivering may well be considered to be substituted for mere muscle tension increase when the latter no longer suffices. This is essentially the idea put forth by Fredericq but which has been ignored by those who have, tacitly at least, used "no shivering" to mean no increase in muscle tension.

In consideration of the gradual and progressive changes in muscle tension which characterize the period preceding visible shivering, the burden of proof as to the existence of other factors would seem to lie with those who are not willing to ascribe to these changes the metabolic increases noted. Cannon points out that Loewy had to assume an increase in muscular tension in many cases where metabolism increased without shivering. A much more improbable assumption would be that increased muscular tension did not exist.

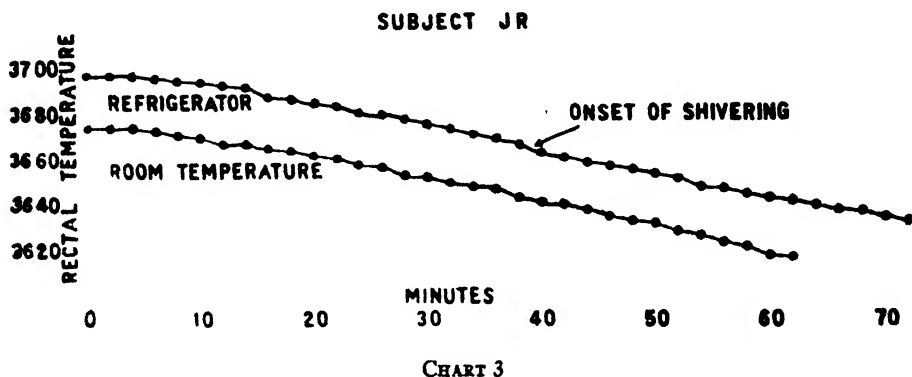
The belief that increased muscular tension is responsible for the increased metabolism which occurs without shivering, does not, of course, exclude the idea of epinephrin production. Cramer (51) believes epinephrin to be important in heat regulation but that its effect is brought about entirely through the sympathetic nervous system. The constancy of the blood sugar, however, argues against epinephrin production.

#### *The Stimulus for the Onset of Shivering*

An effort was made to include in this work a small contribution regarding the stimulus for the onset of shivering. Since the rectal temperatures, taken before and after exposure to cold, had previously shown almost invariably a definite decrease, a few experiments were performed during which a record of the rectal temperature was obtained by means of a resistance thermometer and recording galvanometer. These temperatures, accurate to  $.01^{\circ}\text{C.}$ , were taken every two minutes. The thermometer was inserted a distance of 12 centimeters, this distance being the same in all cases and determined by a bulb of hard rubber fastened to the thermometer. In one experiment the subject lay on a cot in the refrigerator while the rectal temperatures were being recorded. A record of shivering was obtained in the usual manner. In a second experiment, the same subject lay on a cot at room temperature ( $24^{\circ}\text{C.}$ ) during which time the rectal temperature was recorded as above. The data obtained with subject J.R. are set forth in Chart 3. Closely similar results were obtained with another subject.

Two points of importance are to be noted. First, the drop in body temperature, up to the time shivering begins, is the same as obtains when the subject lies down in a warm room. A drop in body temperature occurring on lying down at room temperature has been reported by Benedict and Slack (52). Their results also showed that rectal temperature is an excellent indication of the temperatures of all other parts of the body exclusive of the skin. Second, the slow and gradual drop in body temperature is not lessened by vigorous shivering. From these results it seems certain

that even a slight drop in body temperature can not represent the stimulus for shivering. In both the above cases the shivering was very marked during the last 10 minutes and was taking place when the body temperature had not dropped any more than in the parallel experiment done at room temperature. The above observations illustrate the striking degree of perfection of the heat regulating mechanism. This subject had been in the refrigerator several times before when the metabolism had been determined and it is safe to assume that during the last few minutes of this particular test the heat output was nearly doubled. That the body temperature was not raised the least bit by marked shivering extending over a period of from 15 to 30 minutes, is surprising.



### *Shivering and Skin Temperature*

In an effort to correlate skin temperature change with the onset of shivering, a special thermopile was made to record the surface temperature at various parts of the body. This contact thermopile consisted of four junctions of copper and constantin, and was carefully calibrated.

Benedict and Slack (52) pointed out that surface thermometers are subject to the temperature of the environment as well as to the skin temperature and are "at best unsatisfactory and inaccurate." The unfavorable conditions of use in the work here reported increase still further the uncertainty of the values obtained since the environmental temperature of about 2° C. is very much below that of the body or of the skin. Considerable effort was consequently expended in preliminary trials to find the time that the thermopile should be applied to the skin under the conditions of the experiment. Since the reading on the chart increased only slowly after the thermopile was in contact with the skin for 25 seconds, this was the time of contact used. Readings were taken every two minutes (Chart 4).

The subject sat down in the refrigerator, stripped to the waist, and applied the thermopile according to signals given by a light. As was the case in other experiments, the onset of shivering was not sudden and could not be designated accurately. The definite involuntary movement or twitchings of muscles (usually those about the shoulders) was considered the beginning of shivering. The second test made with subject J.R. followed the completion of the first by an interval of only about 20 minutes, the subject being exposed to a room temperature of 23° C. during this interval. Apparently the skin temperature had not returned to normal, which partly accounts for the lower initial readings in the second test. The thermopile

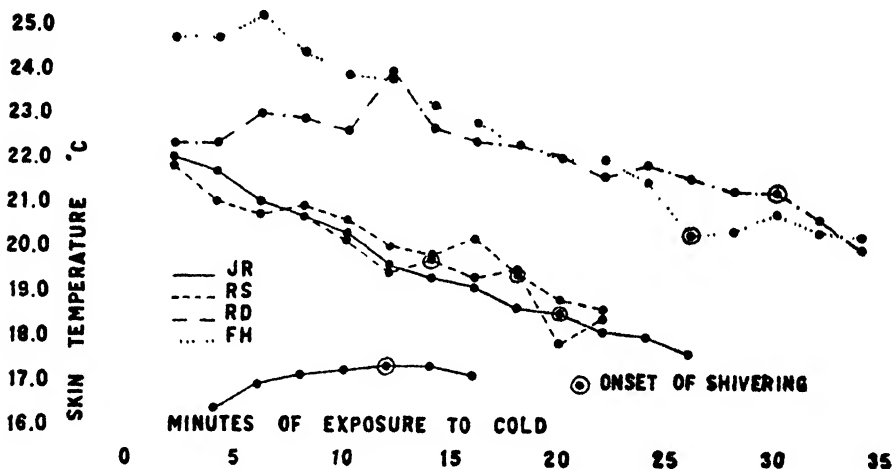


CHART 4

was exposed during this interval to the refrigerator temperature so that the lag of reaching the skin temperature would also tend to make the first readings in the second period low.

The energy metabolism of subjects J.R. and R.D. is shown in Table I. Subject R.D. did not shiver in those experiments as contrasted with subject J.R. and it is significant that the former reached the point of shivering, when stripped to the waist, only after a considerably longer exposure to cold. His skin temperature was apparently somewhat higher when shivering had definitely begun. Emphasis of this small difference, however, is prohibited by the inaccuracies inherent in the method of measurement. It seems reasonable to conclude that when normal persons exposed just previously to room temperature and normally clothed are exposed to cold under the conditions described above, shivering definitely begins when the skin attains a temperature of approximately 19.0° C.

## SUMMARY

A study of the data obtained on 21 human subjects in a basal condition, exposed to an environmental temperature of about 2° C. for one hour and a quarter, permits the following conclusions:

1. There is no change in the protein metabolism corresponding to the increase in energy metabolism.

2. The blood sugar level remains unchanged, indicating that increased epinephrin secretion is not involved.

3. There is no parallelism between the energy metabolism and pulse rate or respiratory rate, but distinct parallelism between heat production and respiratory volume.

4. The reaction to cold in a general way varies inversely as the amount of subcutaneous fat.

5. The increase in energy metabolism considered with the time spent in shivering shows that intense shivering increases the metabolism about 400 per cent.

6. The increase in metabolism shows no relationship to surface area.

7. The heat production is proportional to the amount and the intensity of shivering, and any increase in metabolism not accompanied by definite shivering may be justly ascribed to increased muscular tension.

8. The stimulus for shivering does not consist of even a slight drop in body (rectal) temperature.

9. Shivering begins when the skin attains a temperature of approximately 19° C.

*Acknowledgment*

The writer is pleased to express his thanks to those who so unselfishly volunteered to serve as subjects. Mr. J. W. Karr assisted materially in the careful determination of the blood sugars. It is a pleasure to acknowledge the continual guidance and suggestions of Professor J. R. Murlin.

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## SOME EFFECTS OF RESTRICTED PROTEIN INTAKE ON THE ESTROUS CYCLE AND GESTATION IN THE RAT\*

By

H. R. GUILBERT AND H. GOSS

*(From the Division of Animal Husbandry, College of Agriculture  
University of California, Davis)*

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**T**HE idea that any dietary deficiency may be a limiting factor in reproduction has been developing for a number of years, and experimental evidence, which has been accumulating, supports this conception. Thus Evans and Bishop (3) showed that quantitative undernutrition, induced by limiting the intake of a normal stock diet, seriously interfered with sexual maturity and ovulation in rats.

Deficiencies of single vitamins have been shown, by several workers, to affect reproduction. Inadequate vitamin A impairs the female reproductive system so that fertilization and implantation often fail. In regard to this Evans (6) states,

A level of inadequate vitamin A can be secured, denoted by continuous cornified cell vaginal smears, during which estrus and ovulation occur and are, in fact, fairly frequent. This is only demonstrable by the continuous presence of the male, for no changes occur in the vaginal cell types. Four-fifths of the copulations eventuate in failed implantations. A great many of them fail to establish the condition of pseudopregnancy since another estrus occurs in 5 days.

Atrophy of the testes and ovaries of pigeons results from vitamin B (B & G) starvation according to McCarrison (11) while Parkes and Drummond (12) found that sterility followed quickly in male rats deprived of vitamin B. In female rats vitamin B deficiency results in cessation of estrus and the ovulatory function has been shown to be more sensitive to this lack than general bodily nutrition (Evans and Bishop, 3). That the vitamin B requirement for lactation is greatly in excess of that necessary for optimum growth has been shown by Evans and Burr (7) and by Sure (13).

The well known relation of the fertility vitamin E to reproduction need not be discussed here except to note that sterility of females resulting from this deficiency is not due to impairment of the ovaries or ovulation but to a characteristic disturbance occurring in gestation, which results in the death and resorption of the fetus (Evans & Bishop, 4). Inadequate vitamin

\* The experimental work reported in this paper became coöperative with the United States Department of Agriculture July 1, 1929.

E leads eventually to destruction of the seminiferous epithelium of the male (Evans & Burr, 5).

Evidence is found in the literature that mineral deficiencies may be involved in reproductive disturbances. Naturally occurring deficiency of calcium and phosphorus in the food of domestic livestock has been cited by Tuff (16), who states that experience in badly affected areas of Norway shows unmistakably a relation to reproduction, indicated especially by failure to ovulate. Difficulty in reproduction in cows which was characterized by premature birth of the calves has been reported by Hart and associates (9) and attributed by them to a ration which was deficient in calcium and vitamin A.

Cessation of estrum in cattle under experimental conditions, on rations deficient in phosphorus, was reported by Eckles, Becker, and Palmer (2). Similar observations have been made on cattle grazing phosphorus-deficient veld in South Africa (Theiler, Green and Du Toit, 15), (Du Toit and Bisschop, 1).

Evans and Bishop (3) found that fat-free diets fed to rats interfered with normal ovulation, but no such interference was attributed to carbohydrate-free diets. Qualitative undernutrition with regard to protein resulted in cessation of ovulation.

Our attention has been directed particularly to the effect of phosphorus and protein deficiencies on reproduction, during the course of an investigation on the factors involved in the variable calf crop obtained in range cattle in California. Analyses of range forage collected at different seasons of the year have shown strikingly that most species of forage become deficient in protein and phosphorus during the long drought period, and frequently the ratio of calcium to phosphorus is extremely wide. Since the naturally occurring deficiencies of protein and phosphorus were found simultaneously, an investigation was initiated with rats for the purpose of extending our general knowledge of the independent effects of these deficiencies. The results of experiments on the effects of varying the calcium and phosphorus intake on the estrous cycle and reproduction in the rat have already been reported (Guilbert & Hart, 8).

#### *General Procedure*

The seventy-five female rats used in these experiments were selected at weaning time (21 days of age) and divided into groups. They were kept in metallic cages equipped with false screen bottoms containing 3 meshes to the inch. From one to three rats were kept in each cage.

All of the animals were allowed a normal protein intake until sexual

maturity (about 50 days of age) when some groups were changed to the restricted diets, while other groups were not changed to restricted diets until they had attained an age of about 100 days. It was thus possible to study the effect of diet upon the functioning of ovaries which had been allowed to develop normally.

Following sexual maturity, the stage of the estrous cycle was determined by daily microscopic examination of the vaginal smears.

TABLE I  
COMPONENTS OF THE DIETS  
(grams per 100 grams diet)

	Diet 13	Diet 16	Diet 26	Diet 27	Diet 28	Diet 29	Diet 30	Diet 31
Casein	4.0	—	—	—	15.0	—	—	3.5
Whole milk powder	3.0	3.0	—	—	—	—	—	—
Egg albumin	—	—	10.0	1.0	1.0	1.0	—	1.0
Wheat gluten flour	—	—	10.0	2.0	—	—	—	—
Corn starch	59.2	62.9	55.0	72.0	62.0	76.5	77.5	73.0
Whole wheat	25.0	25.0	—	—	—	—	—	—
Alfalfa leaf meal	—	—	5.0	5.0	—	—	—	—
Butter	5.2	5.2	6.0	6.0	7.0	7.0	7.0	7.0
Cod liver oil	—	—	1.0	1.0	2.0	2.0	2.0	2.0
Wheat germ oil	—	—	1.0	1.0	1.0	1.0	1.0	1.0
Yeast	—	—	—	—	7.0	7.0	7.0	7.0
Yeast extract*	—	—	7.0	7.0	—	—	—	—
CaCO <sub>3</sub>	1.5	1.5	—	—	—	—	—	—
NaCl	0.8	0.8	—	—	—	—	—	—
KH <sub>2</sub> PO <sub>4</sub>	1.3	1.6	—	—	—	0.5	0.5	0.4
Salt mixture	—	—	(4) 5.0	(4) 5.0	(6) 5.0	(6) 5.0	(6) 5.0	(6) 5.0
Crude protein, per cent	7.0	3.5	16.5	4.1	17.5	4.9	4.0	8.2

\* Northwestern dried yeast was first extracted with cold 70% alcohol, acidulated with acetic acid, then with hot 70% alcohol, and the combined extracts evaporated in vacuo to a volume such that 1 cc. was equivalent to 1 gram of yeast.

Three groups of diets were employed in which the sources of protein varied. The per cent of protein in the restricted diets varied from 3.5 to 8 per cent. The composition of the diets is shown in Table I.

Phosphate salts were added to the diets to compensate for the reduction coincident with the reduction of protein, thus maintaining the phosphorus content practically constant. The fat-soluble vitamins were furnished in abundance by butter, cod liver oil and wheat germ oil, or as in the case of diets 13 and 16, by butter and whole wheat. Seven per cent of yeast or

yeast extract is known to supply adequate amounts of the vitamin B complex for growth and ovarian function on diets otherwise poor in vitamin B. Diets 13 and 16 are probably poorest in regard to the vitamin B complex, but the appetites of the animals and the difference in the supply effected by the addition or removal of 4 per cent casein would not appear to account for the difference in results secured on these diets.

<i>Salt 4</i>		<i>Salt 6</i>	
	Per cent		Per cent
NaCl	4.05	NaCl	5.00
MgSO <sub>4</sub>	12.60	MgSO <sub>4</sub>	5.00
Na <sub>2</sub> HPO <sub>4</sub>	23.00	Calcium lactate	29.93
KH <sub>2</sub> PO <sub>4</sub>	17.50	CaCO <sub>3</sub>	26.92
CaCO <sub>3</sub>	9.30	Na <sub>2</sub> HPO <sub>4</sub>	10.10
Calcium lactate	30.80	KH <sub>2</sub> PO <sub>4</sub>	20.00
Ferric citrate	2.70	Ferric citrate	3.00
KI	0.05	KI	0.05
	<u>100.00</u>		<u>100.00</u>

In diets 13 and 16 the protein is derived from milk and wheat, in diets 26 and 27 from egg albumin, wheat and a small amount from alfalfa leaf meal and from the yeast extract, while in diets 28 to 30, the protein is furnished by yeast and egg albumin or yeast, egg albumin and casein.

#### EXPERIMENTAL

A group of nine rats were fed upon the stock diet<sup>1</sup> from weaning until 55 days of age, at which time each rat had had two or more periods of estrum. They were then fed diet 13 containing about 7 per cent protein for a period of 40 days, after which time the protein intake was decreased to 3.5 per cent by replacing 4 per cent of casein by corn starch (Diet 16). The data from this group are summarized in Figure 1, by means of a composite growth curve and a graphical presentation of the estrous cycle history.

There was a slight break in the growth curve upon changing from the stock diet to diet 13, but considering the level of protein fed, the rate of growth was good. Some of the rats missed 1 to 3 periods of estrum immediately following the change of diet but later practically all were having cycles regularly at 4- to 6-day intervals. Upon changing to diet 16 containing about 3.5 per cent protein there was a loss of weight followed by 40 days of maintenance, then a slight decline. Estrus ceased in 5 of the 9 rats within a short time after the change to this diet and the remainder

<sup>1</sup> The stock diet is the McCollum normal diet consisting of, whole wheat 67.5%, casein 15%, whole milk powder 10%, butter 5.2%, CaCO<sub>3</sub> 1.5%, and NaCl 0.8%.

had irregular and long cycles. During the intervals in which there were no manifestations of heat, the vaginal smear exhibited the typical diestrous characteristics.

A group of 11 rats were fed diet 26 (16.5% protein) from weaning time until 105 days of age, at which time they were changed to diet 27 (4% protein). The data from this experiment are shown in Figure 2.

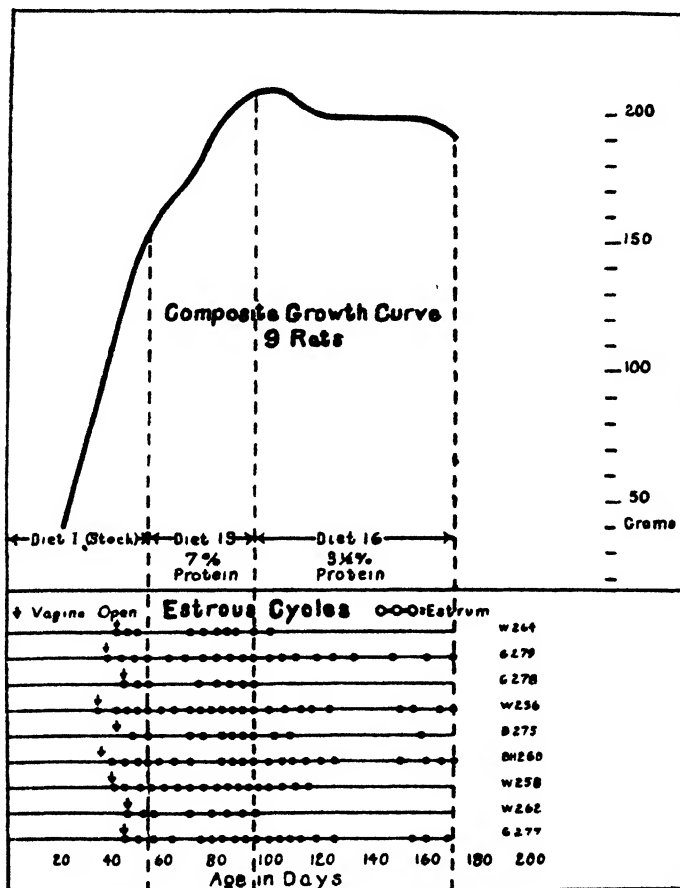


FIG. 1

Diet 26 produced very satisfactory growth, the rats attaining an average weight of 225 grams at 100 days of age. The time of opening of the vagina was somewhat delayed, particularly in 2 of the 11 rats. Upon changing to diet 27 there was an immediate loss of weight, followed by a period of 20 to 30 days in individual rats during which there were slight losses. After

this time, loss in weight was rapid until some of the rats reached a weight only 50 per cent of that attained on diet 26, and were in critical condition. Seven of the 11 rats ceased having estrum within a short period after changing to the low protein diet. The remainder continued for a longer

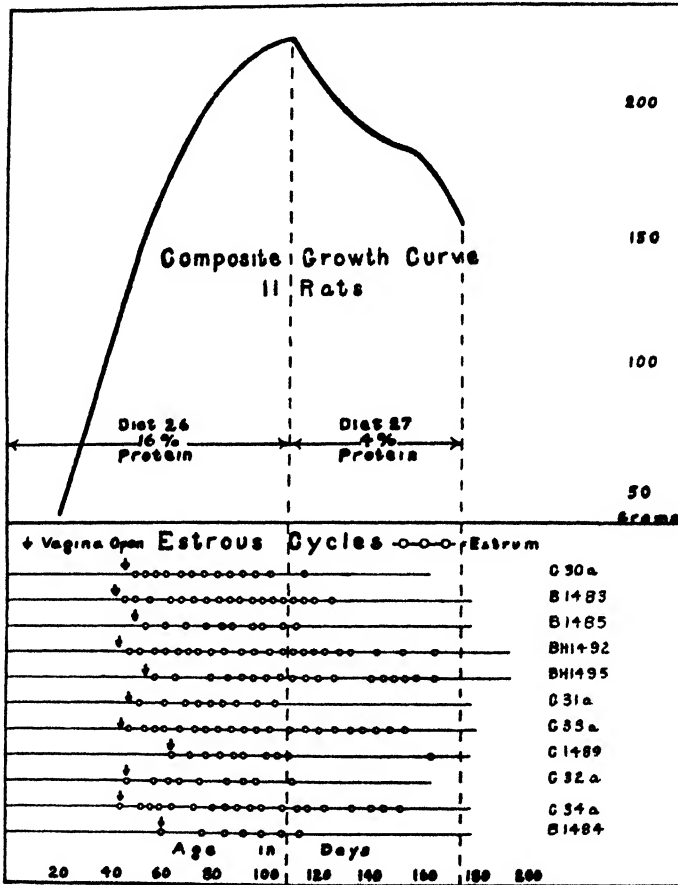


FIG. 2

period as indicated in Figure 2, but all ceased having cycles for a significant length of time before the end of the period.

At the close of the period shown in Figure 2, two rats were autopsied, 4 were changed back to diet 26 and for the remaining 6 rats the protein in the diet was increased by replacing 5 per cent of corn starch with egg albumin. This addition did not lead to improvement of body weight, over a period of 35 days, and the rats were then given diet 26.

There was a rapid recovery in weight on diet 26 in all except one rat which developed pulmonary infection and died. Two individuals did not come into estrum even though they regained or surpassed the weight originally attained on diet 26, and appeared to be in thrifty condition. The total period, prior to autopsy, during which there was no sign of estrus for each of these rats, was 155 days. The remaining rats returned to more or less regular cycles upon regaining the weight lost during the period on the low-protein diet. Several rats began losing hair either shortly before or after being changed from the low-protein diet back to diet 26. New coats of hair developed soon after they began to recover weight on diet 26.

At the close of this recovery test all animals were autopsied. The cessation of the estrous cycles in this experiment was coincident with large losses in body weight, in contrast to a similar cessation of estrus on diet 16 on which the animals maintained their weight at a little under 200 grams and for the most part were fat and apparently in good condition, although there was slightly less protein in their diet.

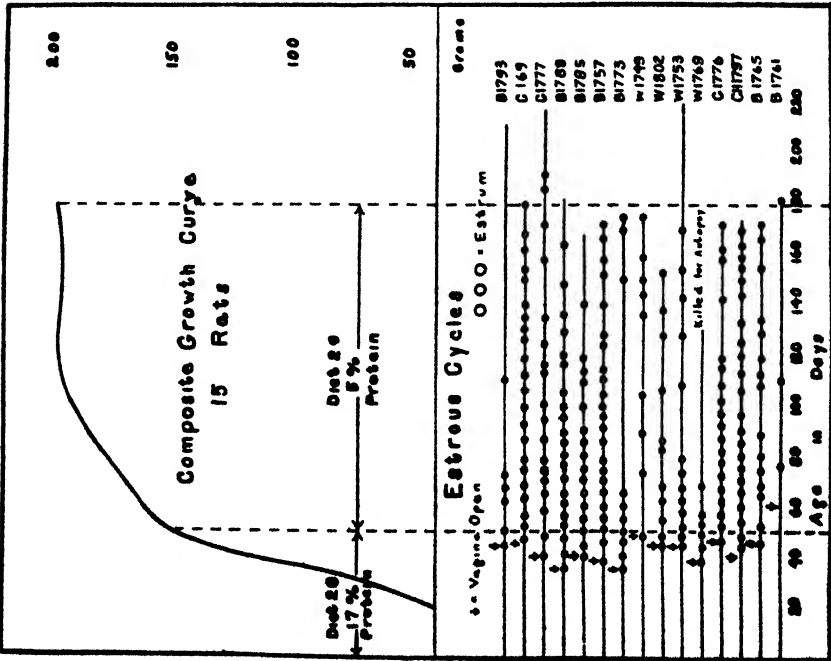
In the third group of experiments fifty-four rats divided into 4 groups were used. Group 1 consisted of 8 rats fed diet 28 throughout the experiment and constituted the controls. Group 2 consisted of 15 rats which were fed diet 28 from weaning time until sexual maturity when they were fed diet 29 which contained about 5 per cent protein. Group 3 consisted of 8 rats which were fed diet 28 from weaning time until 105 days of age, at which time they were changed to diet 29. The reason for the difference in time of changing group 2 and group 3 to the deficient diet was that we wished to ascertain whether or not the effect of deficiency would be greater during a period in which there would normally be rapid growth (50 to 100 days) as compared with practically mature rats. Group 4 consisted of 9 rats which were fed diet 28 from weaning to 105 days of age, at which time they were changed to diet 30 containing about 4 per cent protein. Group 5 consisted of 14 rats which were fed diet 28 from weaning time to sexual maturity, then fed diet 31, which contained about 8 per cent protein. They were continued on this diet until 152 days of age, from which time they were fed diet 30.

The growth of all rats on diet 28 was excellent, the average weight at 100 days of age was about 230 grams, and the estrous cycle was normal. The average interval between periods of estrus for the control group for a period of 160 days from sexual maturity was 5.1 days.

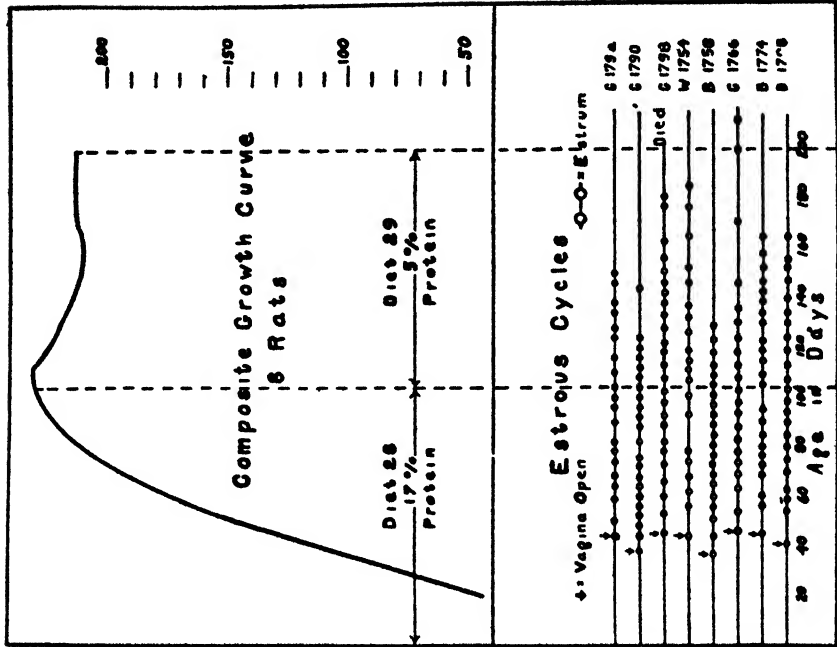
The data for groups 2, 3, 4, and 5 on the restricted diets are presented in Figure 3.

Upon changing from diet 28 soon after sexual maturity to diet 29, con-



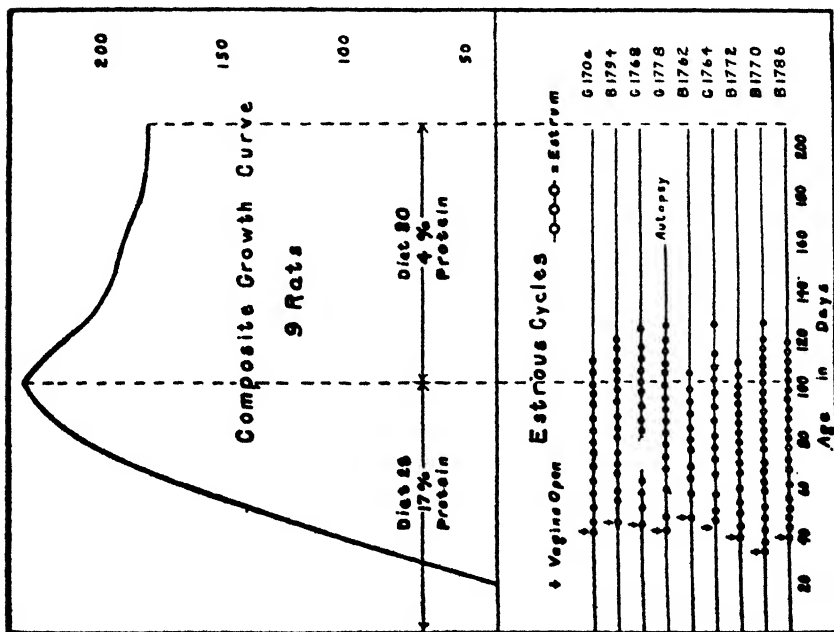


GROUP 2

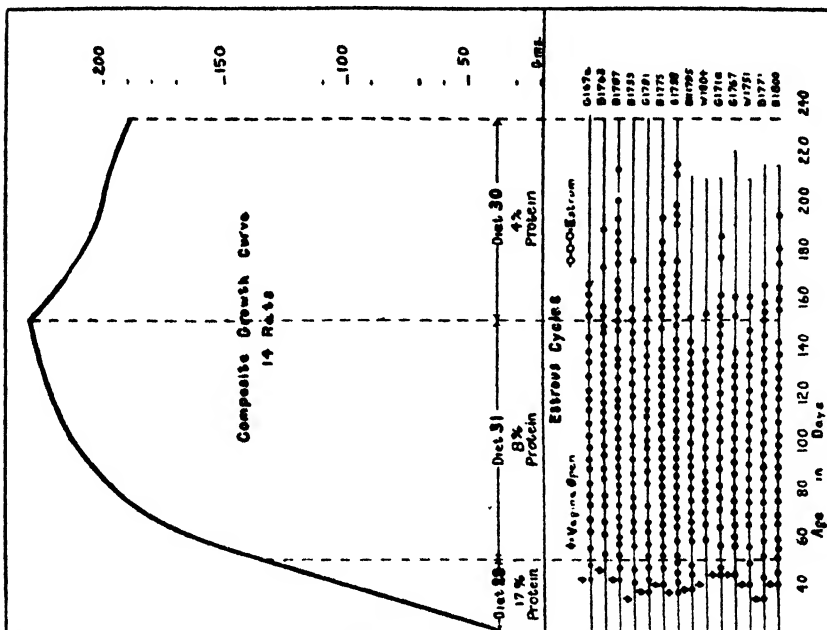


GROUP 3

FIG. 3



GROUP 4



GROUP 5

FIG. 3

taining 4.9 per cent protein, there was a break in the growth curve, but the animals continued to grow until they reached an average weight slightly under 200 grams (see group 2, Figure 3). This weight was maintained throughout the period. The effect of the diet upon estrous cycles was variable. Three rats had practically normal cycles, the remaining 12 rats had irregular, long cycles or ceased entirely. The estrous cycle history of this group was in marked contrast to that of the controls and it is significant that the regularity of the cycle was affected even though the diet permitted growth. Four and nine-tenths per cent of protein from the sources used in diet 29 appears to be marginal in regard to regularity of estrous cycle.

Upon changing group 3 from diet 28 to diet 29 at 105 days of age there was a loss of weight, followed by a long period of maintenance at about an average weight of 210 grams.

The estrous cycle was normal during the period on the control diet and continued with practically normal intervals for from 25 to 60 days after the change to diet 29. Subsequent to this time estrus became irregular or ceased entirely. The effect of the diet on the estrous cycles in this group was not greatly different from that occurring in group 2 which was changed to the low protein diet at sexual maturity. However, it appears that there was somewhat greater persistence of estrus in the younger rats than in the older ones which might suggest that the internal stimulus for ovulation was more pronounced than it was at the later age.

The rats in group 4 which received the control diet until 105 days of age ceased ovulating within 5 to 20 days after being changed to diet 30 containing 4 per cent protein. There was a loss of weight on this diet followed by maintenance at a level of about 180 grams.

The growth rate of the animals on diet 31, containing 8 per cent protein, was only slightly lower than that of the animals on the control diet and these animals were entirely normal in estrous cycle history (see group 5, Figure 3). The effect of reduced protein was again demonstrated in this group when they were changed to diet 30. Eight of the 14 animals ceased having estrus within a short time after the change to this diet and the remainder ceased after a longer period.

*Recovery Experiments:* Four animals from each of groups 3 and 4 were given the control diet (diet 28) at the end of the periods indicated in Figure 3. All gained rapidly in weight, most individuals increasing 30 grams or more during the first 5 days after the change of diet.

With one exception these rats had not been in estrus for from 65 to 105 days, yet they came into estrus in 4 to 6 days following the change of diet.

*Breeding Experiments:* Following the periods indicated in Figure 3, the animals in the control group fed diet 28, and those of group 2, fed diet 29, which came in estrum, were tested for fertility.

Of the control group of 8 females, all were bred one or more times. Four of the females failed to mate during estrum from one to three times before a positive mating was obtained. There were a total of 14 positive matings as shown by examination for plug and sperm in the vagina. Twelve of the 14 positive matings resulted in the placental sign (R.B.C.) at the 13th to the 15th day following coitus. Ten of these pregnancies terminated in litters of from 5 to 12 young which were apparently normal and averaged in weight from 5 to over 6 grams each. Of the two additional pregnancies, one litter of 2 dead rats was delivered 4 days later than the normal time of parturition and the other litter consisted of 7 rats, 5 of which were deformed and were born dead. One positive mating was followed five days later by the recurrence of estrum. Pregnancy was later established in this rat. In one female red blood cells appeared in the vaginal smear on the 12th day following coitus and no litter was born. Thus litters were obtained from all but one rat, and for the most part they were normal in appearance and weight at birth.

It was recognized that 7 per cent of yeast as the principal source of vitamin B in the diet would be deficient for lactation, and an additional 1 to 1.5 grams were fed to each female daily, except in 2 cases, beginning with the 20th day of gestation. However, all except 3 litters died within a few days following parturition, the mothers apparently failing to lactate. Diet 28 was excellent for growth and normal rhythmic functioning of the ovaries, fair for fertility and gestation, but inadequate for lactation even though supplemented with yeast.

In the breeding tests with the animals in group 2, (fed low protein diet, 29) there were 17 instances of failure to breed when during estrum they were placed with a normal male, positive mating never being obtained in several cases. Fourteen positive matings were secured. In 5 of these positive matings, blood appeared in the vaginal smear at the 8th to 12th day following coitus and no litters were born. That pregnancy had been established was shown on autopsy, by the presence of placental cites in the uterus. One animal had red blood cells in the vaginal smear at the 14th day. There was no sign of littering on the 23rd day following mating and there had been a loss of 20 grams in live weight during the preceding 4 days. It was therefore thought that resorption was occurring. The animal was autopsied and 6 live fetuses weighing 2 to 3 grams each were found in the uterus. This was the only case of young being carried alive full time,

resulting from the 14 positive matings in this group. In the other 8 positive matings, fertilization either was not accomplished or else very early death of the embryos occurred, as there was no sign of red blood cells in the vagina, no litters resulted, and either the diestrous smear persisted or the animals came back into estrum within a few days after mating. One animal was bred and came back into estrum and was rebred 5 days later. Red blood cells appeared in the vaginal smear 8 days from the second mating. Four days later the animal was in estrum and mated. This mating was followed by 2 more recurrences of estrus and matings at 5- and 7-day intervals respectively. Estrus occurred more or less regularly for sometime but no further matings were attempted. The repeated matings and recurrence of estrus in this rat together with similar observations on several others indicated that the condition of pseudopregnancy was not induced.

Eight animals from groups 3 and 4 were bred after the recovery period on diet 28. Positive matings were secured usually after 1 to 3 periods of estrum, and they were then given low protein diet 30, during gestation. Of these 8 rats, 2 had blood in the vaginal smear at the 10th day following mating, no litters were born and the animals did not come back into estrum. Five rats had the placental sign (R.B.C.) at the normal time. Two of these died about the time for parturition, autopsy revealing young which were subnormal in weight, in the uterus of each. One rat gave birth to a litter of dead young, 5 days after normal parturition time. One female gave birth, at term, to subnormal young and another female, given stock diet the last five days of gestation, gave birth to living young which were only slightly subnormal in weight. The mating of the remaining animal apparently did not result in fertilization. A female from group 3, diet 29, which was not included in the recovery experiment, was bred. On the 11th day following copulation there was blood in the vaginal smear and what appeared to be an embryo and membranes were removed from the vagina. The animal was autopsied, and four live embryos were found in the uterus. In a fifth placental cite, in the right horn, nearest to the cervix, no membranes or embryo were found, and it appeared that this was the point of origin of the tissues found in the vagina. Histological sections verified that the material found in the vagina was embryological tissue.

In the cases in which red blood cells appeared at the 8th to 12th day following coitus, the vaginal smear was characterized by a more copious flow of blood than the normal placental sign which appears from the 13th to the 15th day, or that of pseudopregnancy. In some cases it amounted almost to hemorrhage at first, and persisted only a few days, while in other cases the bloody smear occurred continuously up to the 23rd day after

ming. This observation, together with autopsy findings, indicates that the death of different individuals in the litter may not occur at the same time.

*Histology of the Ovary:* Upon autopsy of animals which had not been in estrum for 60 days or more, large corpora, and also a large amount of yellow or straw-colored material was observed on the surface of the ovaries. Some follicles were also visible. Although we do not know of data on the length of time for the involution of the corpus luteum in the rat in the absence of estrum, we were surprised to find corpora of comparatively large size persisting. It was computed from the data of Long and Evans (10), in regard to the number of corpora in the ovary of normal rats, that complete involution must occur in about 70 days. We, therefore, sectioned several ovaries to ascertain the character of these corpora and also the nature of yellow pigmented substance. The latter was found to be associated with the amount of interstitial tissue. This was found in greatest amount in individuals which had not been in estrum for 150 days, but was also found in ovaries of females which had not been in estrum for as short a time as 30 days.

In the animals autopsied 60 to 70 days after the last evidence of estrum, as many as 20 to 30 corpora were found in a single ovary, the largest of which varied from 600 to 800 microns or from one-half to two-thirds the size of the normal, full-sized corpus luteum. Some of the corpora were still well vascularized and did not have the profuse connective tissue ingrowth, associated with regressing corpora. Others were poorly vascularized and there was abundant connective tissue. A considerable number of follicles of about one-half mature size, numerous smaller follicles, and comparatively few primordial follicles, were found. Many of the follicles were undergoing atresia.

It is not clear from the data at hand whether there was persistence of the corpora from the last heat periods or whether new corpora were forming without ovulation such as Swezy and Evans (14) describe during pregnancy in the rat.

*Food Consumption:* The question naturally arises as to whether reduced protein did not affect palatability to the extent that the level of food consumption became an important factor in the results secured. Since the food cups employed do not entirely prevent waste, the figures on food consumption include a variable amount of waste, and rats on the deficient diets tended to spill more than the controls. However, when the amount became excessive it was taken into account.

The lowest daily intake per rat was 9.2 grams, and occurred on diet

27 which caused rapid decline in weight. The amount wasted was small, and the daily consumption was only slightly under 5 per cent of body weight. On the other restricted diets the food intake varied from 12.5 to 14.8 grams daily and averaged 6.4 to 7.0 grams daily for each 100 grams live weight. Quantitative undernutrition, therefore, does not appear to have influenced the results in these experiments.

#### SUMMARY

1. Diets containing 3.5 to 5 per cent protein resulted either in cessation of estrus or in long and irregular cycles.
  2. Diets containing 7.5 to 8 per cent protein permitted growth at only slightly less than the normal rate and the estrous cycle was normal.
  3. An inadequate level of protein was secured which permitted estrus in some individuals. These animals were subjected to breeding tests but no litters were produced. The lack of fertility was of 3 general types: (a) Repeated failure to mate when placed, during estrum, with normal males. (b) Apparently infertile matings with recurrence of estrus, the condition of pseudopregnancy not being induced. (c) Fertile matings followed by death of the embryos, the incidence of which was denoted by the appearance of blood in the vagina on the 8th to the 12th day following coitus. One definite case of abortion was recorded.
  4. There was no marked difference in the effect of restricted protein intake on estrous cycle in rats placed upon the deficient diets at 50 days of age, as compared to practically mature rats.
  5. In nearly every case, females which ceased ovulating on deficient diets returned to normal cycles within a short time after being changed back to the control diets.
  6. Food consumption data indicate that quantitative undernutrition was not a factor in the results obtained.
  7. Histological examination of the ovaries revealed as many as 20 to 30 corpora, many of which were one-half to two-thirds the size of a normal, full-sized corpus luteum, remaining as long as 70 days after the last period of estrum. There were also numerous large follicles and also smaller follicles many of which (both large and small) were undergoing atresia. There were few primordial follicles and an abnormal amount of interstitial tissue. It was not clear from the data available whether or not new corpora were forming or whether there was unusual persistence of the corpora formed during the last periods of estrum.
- Acknowledgement:* The authors are indebted to Dr. H. H. Cole for the histological examination of the ovaries.

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# A METHOD OF DETERMINING THE BIOLOGICAL VALUE OF PROTEIN IN THE STUDY OF AVIAN NUTRITION\*

By

J. L. ST. JOHN, O. JOHNSON, J. S. CARVER, AND S. A. MOORE  
(*From the Divisions of Chemistry and Poultry, Agricultural  
Experiment Station, Pullman, Washington*)

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THE determination of the optimum level of protein which should be used in poultry rations and the determination of the relative value of protein supplements from various sources are problems of practical interest to the poultry industry. Comparatively little work of a fundamental nature has been done in attempting to solve problems of protein metabolism in the study of avian nutrition. Mitchell (6) proposed a method for determining the biological value of protein, modified from the method used by Thomas, using the rat as the experimental animal. A more recent discussion of this method is found in a book by Mitchell and Hamilton (10). This method has not previously been modified for use with poultry, partly because of the lack of a satisfactory method for the determination of uric acid in avian excreta. Mitchell (7) calculated the biological value of corn proteins for chickens, but used an assumed value for the digestibility of the nitrogen as a basis for his calculation.

Ackerson, Blish, and Mussehl (1) determined the total endogenous nitrogen metabolism of mature birds without determining the fecal and urinary nitrogen separately. This work was done on individual birds. Later these authors (3) studied the effect of individual variation upon the total endogenous nitrogen metabolism and concluded that it was better to use group averages in studying nitrogen metabolism.

With the method for the determination of uric acid by St. John and Johnson (13) available, a measurement of the so-called "endogenous" and "metabolic" nitrogen elimination (Mitchell, 6, 7, and Mitchell and Hamilton, 10) of growing chicks has been made. These data are essential for the calculation of biological values by the method proposed by Mitchell. Chicks were studied from time of hatching to twelve weeks of age, and the application of these results to a calculation of the biological value of protein in the study of avian nutrition is shown.

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One hundred and fifty S. C. White Leghorn chicks were placed in the batteries described by St. John, Carver, Helphrey, Miller, and Cassel (12) and maintained on the ration, described by them, containing 16 per cent total protein. A group of 20 chicks was selected at intervals of two weeks and placed upon a low nitrogen diet. The chicks were maintained in individual compartments in a separate battery under the controlled conditions previously described by the above authors, and the samples of excreta were collected, preserved, and analyzed by the method described by St. John and Johnson (13). The total nitrogen was determined by the Gunning modification of the Kjeldahl method (sodium sulfate and copper sulfate). The ammonia nitrogen was determined by weighing out 2 grams of the dried sample, adding 5 grams of magnesium oxide and 200 ml. of distilled water and distilling into standard acid at 250 mm. pressure. The temperature of the distilling liquid was about 75 degrees C.

The low nitrogen diet used consisted of starch 63 per cent, sugar 15 per cent, salt mixture 5 per cent, cod liver oil 7 per cent, charcoal 5 per cent, and grit 5 per cent. The per cent of nitrogen in this ration was 0.042.

A careful record of the feed consumption was made and also of the daily weight of each chick throughout the experimental period. A calculation based upon data in the paper by Mitchell, Card and Hamilton (11) shows that the feed consumed by our chicks was sufficient for their energy requirements. The chicks were placed upon the low nitrogen diet for a preliminary period of two days before collection of excreta was begun. The use of charcoal in this diet permitted a judgment of the necessary length of this preliminary period. The primary purpose of the charcoal was to furnish bulk in the diet. It was evident that practically all of the undigested residue from the natural ration upon which the chicks had been maintained had been expelled from the system within less than 24 hours. Therefore a preliminary period of 48 hours gave a sufficient margin of safety within which all of the food nitrogen had been eliminated from the digestive tract of the chicks. This point is discussed again in this paper.

In determining the most suitable procedure to use in this work two lots of chicks (lots three and six) were studied daily. Lot number three was placed on the low nitrogen feed at the age of seven weeks and the daily consumption of feed determined, while each day's excreta was weighed and preserved separately. These chicks were maintained on the low nitrogen feed for twelve days. The data covering this period are presented in Table I.

A number of workers have studied the composition of avian urine, and these results are summarized by Coulson and Hughes (4). A study of their

Table 2 leads one to the conclusion that the data available indicate that approximately 80 per cent of avian urine is composed of uric acid and ammonia nitrogen. Since this is the closest approximation that can be made at present, we have multiplied the sum of the uric acid nitrogen plus ammonia nitrogen by the factor 1.25 (100/80) to calculate the total urinary nitrogen. The amount of fecal nitrogen per day is found by subtracting the total urinary nitrogen from the total nitrogen elimination.

From Table I it is evident that the total weight of dry excreta for lot three progressively decreases from day to day throughout the period up to the last day. After the first day the food intake shows a regular progressive decrease up to the last of the period. The per cent of total nitrogen and of uric acid steadily increases after the first day until the last day of the period. On the other hand when the grams of total and of uric acid nitrogen eliminated per day are calculated, the amount is found to be very nearly constant after the first day. The grams of ammonia nitrogen eliminated per day are not so closely uniform, but the amount is comparatively small so that it has little influence on the total amount of urinary nitrogen. The latter is also about the same in amount from day to day. The fecal nitrogen is comparatively small in amount and rather regularly decreases through the eleven-day period.

Mitchell and Kick (9) assume that metabolic fecal nitrogen is proportional to feed consumption and that the "excretion of fecal nitrogen per kilogram of food consumed on the nitrogen-free ration, . . . measures the excretion of body nitrogen in the feces" on a diet containing nitrogen. Mitchell (8) discusses the justification for this assumption. The weight of fecal nitrogen per kilogram of low nitrogen food intake has been calculated and presented in Table I. There is no consistent trend in the amount eliminated from day to day. The amount of fecal nitrogen eliminated each day per kilogram of body weight does however appear to decrease in the latter part of the period. The urinary nitrogen elimination per kilogram of chick weight shows a slight tendency to increase throughout the eleven-day period.

Checking differences occurring between each of the first three days in the various columns in Table I shows that in many cases there is more difference between the first and second day than between the second and third and subsequent days. This confirms the indication evident from the use of charcoal in the diet and makes it clear that a two-day preliminary period is sufficient to allow the avian system to adjust itself to the endogenous level of nitrogen excretion. In fact it appears from the data and from the evidence based on the use of charcoal in the low nitrogen ration that a one-day preliminary period would probably be sufficient.

TABLE I  
ENDOGENOUS AND METABOLIC NITROGEN OF CHICKS BETWEEN SEVEN AND NINE WEEKS OF AGE (Lot 3)

Day	Total weight of chicks av.	Food intake	Total dry excreta	Total N	Uric acid	NH <sub>3</sub> N	Total uric acid N	Total NH <sub>3</sub> N	Uric acid N+NH <sub>3</sub> N	Urinary N	Fecal N	Fecal N per kg. food intake	Fecal N per kg. weight per day	Urinary N per kg. of chick wt.
	gm.	gm.	gm.	%	%	%	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
1	5210	623	168.7	2.93	4.03	.118	2.27	.20	2.47	3.09	1.85	2.97	.355	.59
2	5304	660	115.2	2.39	3.55	.096	1.36	.11	1.47	1.84	.91	1.38	.172	.35
3	5238	500	105.1	2.55	4.07	.072	1.43	.08	1.51	1.89	.79	1.58	.151	.36
4	4948	350	94.5	2.98	4.63	.028	1.46	.03	1.49	1.86	.96	2.74	.194	.38
5	4734	271	73.7	3.74	6.33	.132	1.56	.10	1.66	2.08	.68	2.51	.144	.44
6	4465	159	62.6	4.38	7.00	.222	1.46	.14	1.60	2.00	.74	4.65	.166	.45
7	4273	133	49.7	4.97	8.73	.208	1.45	.10	1.55	1.94	.53	3.88	.124	.45
8	4086	123	46.8	5.84	11.78	.148	1.84	.07	1.91	2.39	.34	2.76	.083	.58
9	4005	116	42.2	6.18	12.75	.158	1.79	.07	1.86	2.33	.28	2.41	.070	.57
10	3706	81	38.0	6.46	12.86	.124	1.63	.05	1.68	2.10	.35	4.32	.094	.57
11	3610	95	27.0	8.09	17.29	.116	1.56	.03	1.59	1.99	.19	2.00	.053	.55
12	3164	71	29.6	7.95	15.89	.142	1.57	.04	1.61	2.01	.34	4.79	.107	.64

TABLE II  
ENDOGENOUS AND METABOLIC NITROGEN OF CHICKS BETWEEN ELEVEN AND THIRTEEN WEEKS OF AGE (Lot 6)

Day	Total weight of chicks av.	Food intake	Total dry excreta	Total N	Uric acid N	NH <sub>3</sub> N	Total N	Total uric acid N	Total NH <sub>3</sub> N	Uric acid N+NH <sub>3</sub> N	Urinary N	Fecal N	Fecal N per kg. food intake	Fecal N per kg. weight of chick per day	Urinary N per kg. of chick weight
	gm.	gm.	gm.	%	%	%	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
1	13351	1511	459.1	4.03	6.96	.116	18.50	10.65	.53	11.18	13.98	4.52	2.99	.339	1.05
2	13578	1684	676.9	4.14	6.90	.096	28.02	15.57	.65	16.22	20.28	7.74	4.60	.570	1.49
3	13180	1180	389.5	3.48	5.47	.092	13.55	7.10	.36	7.46	9.33	4.22	3.58	.320	.71
4	13000	1037	205.2	2.97	3.66	.092	6.09	2.50	.19	2.69	3.36	2.73	2.63	.210	.26
5	12621	1095	300.3	2.38	2.90	.060	7.15	2.90	.18	3.08	3.84	3.30	3.01	.261	.31
6	12247	644	183.8	3.73	4.45	.096	6.86	2.73	.18	2.91	3.64	3.22	5.00	.263	.30
7	11976	252	227.0	3.09	4.63	.074	7.01	3.50	.17	3.67	4.59	2.42	9.60	.202	.38
8	11320	491	163.7	3.79	6.20	.144	6.20	3.38	.24	3.62	4.53	1.67	3.40	.148	.40
9	10808	362	112.7	5.52	8.48	.200	6.22	3.19	.23	3.42	4.28	1.94	5.36	.179	.40
10	10599	317	107.9	5.63	11.27	.248	6.07	4.05	.27	4.32	5.40	.67	2.11	.063	.51
11	12378	1411	443.8	3.82	5.08	.116	16.95	7.52	.51	8.03	10.04	6.91	4.90	.558	.81
12	13228	1559	586.8	3.70	5.44	.124	21.71	10.64	.73	11.37	14.21	7.50	4.81	.567	1.07
13	13346	1433	655.7	3.50	5.37	.112	22.95	11.74	.73	12.47	15.59	7.36	5.14	.551	1.17
14	14078	1491	601.3	3.56	5.77	.112	21.41	11.57	.67	12.24	15.30	6.11	4.10	.434	1.09
15	14738	1905	700.2	3.56	5.60	.096	24.93	13.07	.67	13.74	17.18	7.75	4.07	.526	1.17
16	15183	1891	735.9	3.70	6.25	.064	27.23	15.33	.47	15.80	19.75	7.48	3.96	.493	1.30

Low

nitrogen

ration.

Commercial

ration.

An additional lot of chicks (lot 6) was started on the experimental ration at the age of eleven weeks. To obtain a more complete picture of the response of the chicks to a low nitrogen diet, it seemed desirable to study the nitrogen elimination on the natural ration for several days preceding and following the feeding of the low nitrogen diet. The excreta were collected daily for two days before the birds were put on the low nitrogen diet, then for the two-day preliminary period, followed by daily collection for a six-day experimental period (making two three-day periods). Finally the birds were again placed on the natural ration containing added dry skim milk at a 16 per cent protein level and samples were collected daily for an additional six days. The data are presented in Table II.

The per cent of total nitrogen in the dry excreta decreases as soon as the chicks are put on the low nitrogen diet and then increases until they are again placed on the natural ration when the percentage again decreases and continues at a fairly constant level. The total nitrogen elimination immediately decreases with the feeding of the low nitrogen diet and continues at about the same level, then quickly increases to about the former level when the chicks are returned to the nitrogen-containing ration. The per cent of uric acid and total uric acid elimination follow practically the same trend as does the total nitrogen. The same may be said of the ammonia and total urinary nitrogen figures and the urinary nitrogen elimination per kilogram of weight. However, the fecal nitrogen elimination continues to decrease throughout the period of low nitrogen feeding before it rises to the previous level. The grams of fecal nitrogen per kilogram of food intake vary from day to day throughout the total sixteen-day period, although there is a slight tendency for the amount during the low nitrogen period to be below the amount for the remainder of the period. The data for the fecal nitrogen elimination per kilogram of body weight follow in trend those for the total fecal nitrogen.

Other lots of chicks were started at the ages of two, four, nine, and ten weeks. In the case of the first two lots, the collection of excreta samples was made in four-day periods with a four-day preliminary period, while for the rest, on the basis of the data presented above, the periods were three days in length, daily samples being preserved separately in the case of lots three and six. The diet for the first two lots was slightly different from that subsequently used. Data for the second half of lot two are incomplete. The data for lots 1, 2, 4, and 5 are not presented in full but a summary of the data for all six lots is given in Table III. The figures given in this table for lots three and six are averages of the results for each day of the two three-day periods following the preliminary period. The first

and second halves of the period for each lot are tabulated separately in Table III. The fecal nitrogen both per unit of food and per unit of weight are given. The total endogenous nitrogen elimination is also given.

The fecal nitrogen per kilogram of feed intake varies between lots of birds but there is no trend either in the first or second half of the period.

TABLE III  
FECAL, URINARY, AND TOTAL NITROGEN ELIMINATION OF CHICKS AT DIFFERENT AGES  
ON A LOW NITROGEN RATION

Lot	Age at be- gin. of period	Length of period	Fecal N per kg. food in- take	Fecal N per kg. weight per day	Urinary N per kg. weight per day	Total N elimination per kg. per day
No.	weeks	days	gm.	gm.	gm.	gm.
(First half of period)						
1	2	4	3.30	.149	.52	.669
2	4	4	1.29	.048	.49	.538
3	7	3	2.28	.163	.39	.553
4	9	3	4.07	.253	.33	.583
5	10	3	2.15	.139	.37	.509
6	11	3	4.00	.242	.33	.572
Av. of all lots			2.85*	.166	.405	.57
(Second half of period)						
1	2	4	2.72	.068	.63	.698
3	7	3	3.80	.124	.49	.614
4	9	3	4.09	.135	.42	.555
5	10	3	3.21	.099	.65	.749
6	11	3	3.62	.130	.44	.570
Av. of all lots			3.49	.111	.526	.637
Av. all lots, both halves			3.14	.141	.460	.601

\* Calculating this value to a dry matter basis gives 3.06 grams of fecal nitrogen per kilo. of dry matter in the food intake. There was 6.81 per cent of moisture in the low nitrogen diet.

Mitchell (6) found "unaccountable variations" in the amount of metabolic fecal nitrogen eliminated by rats when this value was determined at successive separated intervals. The average for the second half of the period is larger than that for the first half, but on comparing the first and second half of the period for each lot, it is questionable if this higher average is significant. If the low value for lot two in the first half is omitted, the



average for the five remaining lots is 3.16 grams. The fecal nitrogen per kilogram of body weight appears to be significantly lower in the second half of the periods since not only the average is lower, but the second half is lower for each lot than the first half. Similarly with the urinary nitrogen it appears higher in the second half both in average and for each lot. In none of the data presented in Table III does it appear that there is a variation of either fecal or urinary nitrogen due to differences in the age of the chicks within the twelve-week period covered by this work. Since it would be expected that the chicks would be more nearly normal in the first part of a low nitrogen feeding period than in the latter part, and in view of the facts stated above, the averages for the first half have been accepted for use in the calculation of biological values.

A few determinations of total nitrogen elimination (endogenous plus metabolic nitrogen) have been made on birds from  $4\frac{1}{2}$  months to 2 years of age by Ackerson, Blish, and Mussehl (1, 2, 3). Their results vary from 325 to 115 mg. per kilo. per day depending upon the age of the birds. The total nitrogen excreted on a low nitrogen diet decreased as the birds aged. The results for total nitrogen eliminated reported in Table III above, average 570 mg. per kilo. for the first half of the experimental periods. This is 75 per cent above the highest result reported by Ackerson, Blish, and Mussehl and extends the curve for variation with age which their data suggest. They did not determine uric acid and therefore do not report data for endogenous and metabolic nitrogen separately. Also the results for urinary nitrogen reported in Table III above are within the range found by Hephrey (5).

Mitchell (6) states that "rats will ordinarily excrete from 1.5 to 3.0 mg. of nitrogen per gram of nitrogen-free ration consumed." From the context it is understood that he means "metabolic" nitrogen. This agrees closely with the results for chicks reported in Table III. Mitchell gives the amount of urinary nitrogen on a low nitrogen ration in his tables 14 and 15. An average of these data shows 0.212 grams of urinary nitrogen per kilo. of body weight. Judging by weight, the rats used by Mitchell were between four and twelve weeks of age. The average value of 405 mg. of urinary nitrogen per kilo. of body weight shown in Table III above, for chicks, is approximately twice the result obtained by Mitchell for rats.

As an example of the method of calculation of biological values, the data for the fourteenth day of lot 6 (Table II) is used. There were twenty chicks in the lot. The nitrogen intake is calculated from the food intake and in this case is  $38.32 (1491 \times 0.0257)$  grams for the twenty chicks, for this day. The total urinary and fecal nitrogen are given in Table II. The

metabolic nitrogen elimination is computed from the food intake and the average metabolic nitrogen level given in Table III ( $1.491 \times 2.85 = 4.25$ ) while the endogenous nitrogen elimination is determined from the total weight of the twenty chicks and the average endogenous nitrogen per kilogram per day given in Table III ( $14.078 \times 0.405 = 5.70$ ). We then have,

	grams
a = N intake ( $1491 \times .0257$ )	38.32
b = Total N elimination	21.41
c = Total Urinary N	15.30
d = Fecal N (b - c)	6.11
e = Metabolic N ( $1.491 \times 2.85$ )	4.25
f = Food N in feces (d - e)	1.86
g = Endogenous N ( $14.078 \times .405$ )	5.70
h = Food N in urine (c - g)	9.60

The twenty chicks then received daily  $38.32 - 1.86 = 36.46$  grams of absorbed nitrogen. The food nitrogen found in the urine which was wasted in metabolism is the difference between the total urinary nitrogen elimination and endogenous nitrogen and in this case equals  $15.30 - 5.70 = 9.60$  grams. It is then evident that  $36.46 - 9.60 = 26.86$  grams of nitrogen were retained in the body. Since the biological value as defined by Karl Thomas and by Mitchell is the per cent of absorbed nitrogen which is retained in the body, it is equal in this instance to  $100 (26.86 \div 36.46) = 73.67$ . Reduced to a formula the biological value

$$100 \times \frac{[a - (\{b - c\} - e)] - (c - g)}{[a - (\{b - c\} - e)]} =$$

$$100 \times \frac{[38.32 - (\{21.41 - 15.30\} - 4.25)] - (15.30 - 5.70)}{[38.32 - (\{21.41 - 15.30\} - 4.25)]} = 73.67$$

If the value for the metabolic nitrogen per kilogram of chick weight (0.166) is used in place of the method of calculation used above, the biological value is found to be 72.21.

#### SUMMARY

The endogenous and metabolic nitrogen elimination of S.C. White Leghorn chicks has been studied from time of hatching to twelve weeks of age. Twenty chicks were used in each lot studied. The urinary nitrogen was calculated from the sum of the uric acid nitrogen and ammonia nitrogen elimination by multiplying by 1.25. The per cent of total nitrogen and

uric acid eliminated varies from day to day but the total amount per day is nearly the same throughout the period. The total fecal nitrogen is comparatively small in amount and decreases somewhat during the nitrogen-free period. The fecal nitrogen per kilogram of food intake varies from day to day, but remains at about the same level throughout the period. Calculated per kilogram of body weight, the fecal nitrogen decreases in the latter part of the period. The response of chicks to a low nitrogen diet and to the resumption of a commercial ration is shown.

Within the twelve-week period following hatching no variation of either fecal or urinary nitrogen was found which correlated with the age of the chicks. The averages for the six lots were accepted for use in calculating biological values.

A method of calculating the biological value of protein for use with poultry is presented.

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# THE UTILIZATION BY HUMAN SUBJECTS OF THE NITROGEN, CALCIUM, AND PHOSPHORUS OF THE NAVY BEAN (*PHASEOLUS VULGARIS*) WITH AND WITHOUT A SUPPLEMENT OF CYSTINE\*

BY MARTHA S. PITTMAN

*(From the Nutrition Laboratory of the Department of Home Economics  
and Household Administration of the University of  
Chicago, Chicago, Ill.)*

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PRAUSNITZ (14) reported the results of a digestion experiment on one man who ate daily for 3 days a diet consisting of 500 grams of navy beans and a liter of beer. Since only about 70 per cent of the beans were digested by his subject, Prausnitz concluded that they were not used economically by the body and recommended moderation in employing them in the diet.

A further attempt was made by Snyder (24) to determine the nutritive value of navy beans. He also studied the results of removal of the skins, of cooking the beans with and without the addition of baking soda, and particularly their effect upon the digestibility of other foods. The beans were baked and fed to active young men. For one set of experiments they were eaten with a simple diet of bread and milk, some of the subjects consuming as much as 500 grams of beans daily. The coefficients of digestibility for the protein on this diet appeared to vary greatly with individuals, ranging from 76 to 87 per cent with an average of 80 per cent. With the addition of butter or oleo to the diet the coefficients were higher. If the skins were not removed, 25 per cent less protein was digested and the loss was still greater if the soda were omitted in the cooking process. As a result of these experiments, Snyder also recommended a moderate use of beans in the diet—not to exceed 120 grams of raw beans daily—and stressed the value of proper combinations and preparation.

Wait (25) performed similar experiments using young men as subjects. Besides navy beans, his diets included pork for seasoning, bread, butter, bananas, and sugar. He wished to approximate a mixed diet believing it to be more favorable to digestion. When beans were used in quantities ranging from 375 to 438 grams daily, Wait obtained coefficients of digesti-

\* The experimental data in this paper are taken from a dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Ogden Graduate School of Science in the University of Chicago, 1930.

bility of 73 to 78 per cent for the protein. With only 150 to 175 grams daily, the coefficients were slightly higher, varying from 78 to 81 per cent. He therefore agreed with the previous workers as to the amounts to be eaten and suggested that beans, as he used them, are less completely digested than other common foods.

The more recent literature on the subject deals largely with animal experiments. McCollum, Simmonds, and Pitz (7) found that when beans were used as the sole source of protein for rats, they appeared to be of low nutritive value resulting in stunted growth and a high mortality rate. These authors therefore recommend that navy beans be supplemented by other proteins of better quality and, because of digestive disturbances, that they be used in moderation.

Johns and Finks (4) were unable to obtain good growth in rats with navy beans until the latter were cooked and cystine was added in the proportion of 2 per cent of the protein by weight. They suggest that heating with water may cause some molecular rearrangement which accounts for the greater digestibility or that it may destroy some toxic quality.

Mitchell (9) obtained with rats at a 10 per cent level of intake, the low biological value of 38.4 per cent for the protein of navy beans after steam cooking. He defines the term "biological value" as the percentage of absorbed nitrogen that is not eliminated in the urine. In similar experiments with other foods at this level, milk proteins averaged 84.7 per cent; potato proteins, 66.7 per cent; oat proteins, 64.9 per cent; and corn proteins, 59.6 per cent.

The possible effect of crude fiber on the excretion of nitrogen, calcium, and phosphorus has been recognized for some time. Since navy beans are relatively high in fiber, this point is to be considered when they play an important part in the diet. Mitchell (10) working with rats which received varying amounts of filter paper in a protein-free diet, found fecal nitrogen increased as much as 42 per cent with this type of roughage. Willard, Whitacre, and Blunt (26) comparing results of feeding human subjects two different diets—one apparently higher in fiber than the other—noted lower coefficients of digestibility for the nitrogen in the high-fiber diet.

In contrast to this view, Sjollema (23) observed that increases in indigestible fiber in the diet of rabbits had little effect on nitrogen output. However, the increased fiber did cause a higher fecal loss of calcium and, to a lesser degree, of phosphorus.

Another factor affecting the usage of calcium and phosphorus may be the amount of carbohydrate in the diet. Bergeim (1) reported, as a result of experiments with rats, that when lactose furnished from 25 to 50 per

cent of the calories the absorption of calcium and phosphorus was improved. This was particularly true of calcium. Such carbohydrates as glucose, maltose, and starch, had little or no effect. Bergeim attributed the superiority of lactose to its tendency to form lactic acid in the alimentary tract which is believed to increase the solubility of calcium phosphate. Earlier work indicated that large quantities of glucose and sucrose taken in concentrated form depressed hydrochloric acid secretion making conditions unfavorable to the absorption of calcium and phosphorus.

#### EXPERIMENTAL PROCEDURE

Because of the unfavorable reports concerning navy beans for human food, an attempt was made to determine their efficiency as the chief source of nitrogen in a diet providing the essentials for nutrition as recognized today. In doing this the conditions were also unusually favorable for observing the utilization of the calcium and phosphorus. The beans were prepared in two different ways and supplemented with cystine in some cases to determine if the addition of this amino acid, which is considered the chief limiting factor in navy beans, would increase their utilization.

The experiment was divided into two main parts. In the first series of investigations the beans were eaten as a thick purée; in the second, they were baked. These will be designated respectively as the Bean Purée and the Baked Bean Series. Each of these was subdivided as follows:

##### Without added cystine

Preliminary period	3 days
Collection period	10 days (2 periods of 5 days each)

##### With added cystine

Preliminary period	3 days
Collection period	10 days (2 periods of 5 days each)

**Subjects.** Five healthy adult women served as subjects, three being used for each phase of the investigation. All were university students engaged in somewhat similar activities involving more or less laboratory and class room work.

**Diet.** The diet consisted of navy beans, purified butter fat, sucrose, lactose, grape juice, and lemon juice. The quantities used are shown in Table I. Except for Vitamin D it was doubtless adequate unless the quality of protein should prove to be poor.

The protein was reckoned on the basis of 80 per cent of the requirement set by Sherman of 44.4 grams per 70 kilograms of body weight. This low

level was used to permit differences due to the factors under investigation to be detected readily. The beans were used in sufficient quantity to supply from 90 to 94 per cent of the nitrogen, 80 to 85 per cent of the calcium, and from 84 to 95 per cent of the phosphorus of the various diets. The desired number of calories for the different subjects was secured by varying the amounts of fat and sugars consumed.

TABLE I  
AMOUNT OF FOOD EATEN BY THE DIFFERENT SUBJECTS

Food	Subject			Subject		
	A	B	C	C	D	E
	gm.	gm.	gm.	gm.	gm.	gm.
Beans, dry	147.5	119.5	138.0	113.8	163.1	120.9
Butter fat	55.5	38.9	44.4	55.5	99.9	55.5
Sucrose	106.3	106.3	81.3	100.0	112.5	100.0
Lactose	68.5	50.0	50.0	50.0	75.9	50.0
Grape juice	600.0	600.0	600.0	600.0	600.0	600.0
Lemon juice	128.2	128.2	128.2	128.2	128.2	128.2

TABLE II  
DIET AS CALCULATED FOR THE VARIOUS SUBJECTS

Subject	Protein	Calcium	Phosphorus	Calories per kg.	Total calories	Excess N. base*
	gm.	gm.	gm.			cc.
Bean purée series						
A	33.2	0.333	0.774	36.0	2358	55.22
B	26.9	0.288	0.642	38.0	2037	50.17
C	31.0	0.318	0.729	37.7	2051	53.50
Baked bean series						
C	25.6	0.279	0.615	42.4	2143	49.14
D	36.7	0.358	0.847	39.6	2863	58.02
E	27.2	0.290	0.648	40.4	2167	50.42

\* Without added cystine.

Distilled water was used for all purposes. It was taken *ad libitum* during the Bean Purée Series and the amount consumed varied considerably with the individual and the weather. In the Baked Bean Series each subject took approximately 1200 cc. daily in addition to that obtained in the food. This included a small cup of black coffee for two of the subjects each morning.

In the latter half of each series, 1-cystine, in the proportion of 2 per cent of the weight of the calculated protein, was added to the diet. The allowance for each day was sprinkled over the beans at the time of eating. The amount of salt used (C. P. grade) was not controlled but in no case was it excessive.

The beans were purchased in two lots, one for each series of experiments. The day's supply for each individual was prepared separately on the day before it was to be used. After soaking over night, the beans for purée were salted, and brought to the boiling point over the direct heat, after which they were covered and cooked until soft. This required from 4 to 5 hours in a regulated oven set at 250° F. They were then run through a sieve and all pulp and washings returned as completely as possible, so the loss in preparation was negligible. They were divided into 3 portions, one for each meal. The butter fat was added when they were reheated for serving.

The beans for baking, after soaking, were put into individual baking dishes, one for each meal, and baked with the salt at 350 to 375° F. for 10 hours. The butter fat was added for the last 30 minutes of baking.

The remainder of the diet was combined into a fruit punch. The three meals a day therefore consisted merely of beans and punch.

The amount of roughage in the diet was limited to that present in the beans. The acid-base balance was the same for both periods without added cystine. The excess base was unusually high and, even after cystine was added, the diet was still strongly basic.

The calories remained the same for each subject in both series except for the small increase due to the cystine in the latter half of each period. Because of some loss of weight by the subjects during the Bean Purée Series, which averaged 36 to 38 calories per kilogram, the calories were increased in the Baked Bean Series ranging from 39.6 to 42.4 per kilogram for the various subjects.

*Samples.* The usual precautions were observed in the collection and handling of samples. Aliquot portions of the foods were saved each day. The beans were cooked, dried at 80° C., ground, and thoroughly mixed. The fruit juices were combined and preserved by canning by the cold-pack method using a water bath. Butter fat, sucrose, and lactose were not sampled as they were considered 100 per cent pure and therefore no analyses were thought necessary.

The urine was preserved with toluol and hydrochloric acid. Each day's collection was kept separate until after nitrogen determinations were made. The feces were mixed with acidified alcohol before drying. A composite was made for each 5-day period.



**Methods for Analysis.** The pH of the urine was obtained colorimetrically. Total nitrogen was determined by the Kjeldahl-Gunning procedure. Calcium was obtained volumetrically by a modified McCrudden method (8) using Shohl's (20) and Shohl and Pedley's (21) suggestions for controlling pH. Total phosphorus was determined gravimetrically by the Neumann method (12) as modified by Lundell and Hoffman (5) and McCandless and Burton (6). Accuracy of technic was proven by analysis of materials of known composition.

## RESULTS AND DISCUSSION

The subjects maintained good health throughout the experiment with the exception that mild indigestion, apparently due to formation of gas, was reported by two subjects in the Bean Purée Series. This difficulty disappeared after the first few days. Since one explanation for such gas is the action of bacteria on fiber, more trouble was expected in the Baked Bean Series in which the fiber was not finely divided. However no trouble occurred, perhaps because of the longer cooking period of the baked beans or the fact that the purée was eaten in hot weather when beans are less appetizing.

It was believed the diet supplied an abundance of calories, yet a slight loss of weight occurred with all subjects. It is possible that with the high-fiber diet used in this experiment less food was absorbed than was expected, making the calories insufficient. However, increased calories in the Baked Bean Series did not decrease the loss in weight, so some other factor was doubtless involved.

The diet was deficient in Vitamin D but experimental work indicates that lack of this vitamin in the diet of normal adults for so brief a time as that covered by this investigation would not affect the results materially.

**Urine Findings.** The pH of the urine varied greatly with individuals and also in the same individual from day to day even though the diet was practically constant in every respect. The urine seemed to increase in acidity when the weather was warm. The addition of cystine lowered the potential alkalinity of the diet but it did not always result in a more acid urine.

Although some exceptions occurred, the average pH of the urine was more acid than the 6.64 suggested by Hawk and Bergeim (3) for a vegetable diet. The explanation may be, as offered by Pickens and Hetler (13), that although the basic elements are high in grape juice they do not increase the alkalinity of the urine as expected.

**Feces Findings.** The daily wet weight for the feces ranged from 64.3 to

174.7 grams a day. The average for all periods and subjects was 123.7 grams a day which was equivalent to 25.9 grams dry weight. The average amount of residue, with some exceptions, was not so high as was anticipated because of the fiber in the beans. In accordance with expectations, it was slightly lower in the Bean Purée Series where the beans were broken by running through a sieve.

*Nitrogen Findings.* In these experiments no corrections were made for endogenous nitrogen. However, the results appearing in Tables III and IV are believed to indicate the efficiency of the navy bean as a source of nitrogen as used in these experiments.

The loss of nitrogen in the feces for both series of experiments ranged from 23 to 43 per cent with an average of approximately 34 per cent. In the Bean Purée Series the excretion of nitrogen in the feces varied from 1.10 to 2.71 grams with an average for all subjects of 1.87 grams. In the Baked Bean Series the excretion ranged from 1.45 to 2.41 grams and averaged 1.93 grams. It is thus seen that the loss was practically the same for both methods of cooking. The large amount of fiber in the beans probably explains such high fecal nitrogen on a diet averaging only 5.2 grams of nitrogen per capita per day.

The average coefficient of digestibility ranged from 57 to 77 per cent for the the various subjects. Both of these extremes were obtained on the Bean Purée diet. The average coefficient of digestibility was 65.9 per cent for all subjects for both periods, so no difference can be attributed to the method of cooking.

In contrast to this, Wait (25) reported an average coefficient of 78 per cent for the nitrogen of white beans used in a mixed diet while Mitchell (9) obtained for rats on a bean diet a value of 76 per cent after correction had been made for metabolic nitrogen in the feces. The lower coefficients obtained in this investigation probably are explained to a large extent by the high crude fiber content of the diet. The fact that the beans were eaten in fairly large quantities, furnishing approximately 20 per cent of the calories consumed, may also have been a factor. It may explain too, a somewhat similar value of 70 per cent obtained by Prausnitz (14) when his subject subsisted entirely upon beans and beer. This harmonizes also with Snyder's higher figures of 80 per cent when some milk was used and of 88 to 90 per cent when a greater variety of foods was eaten.

It appears that the nitrogen from the beans, supplemented by the very small quantities obtained from the remainder of the diet, was inadequate to maintain the body in nitrogen equilibrium for any length of time. The nitrogen intake ranged from 4.23 to 6.73 grams per day or 0.082 to 0.093

TABLE III  
NITROGEN FINDINGS  
Bean Purée Series

Subject	Expt.	Period	Average daily intake				Average daily output					Balance
			Beans	Other foods	Cystine	Total	Urine	Feces	Total	Urine	Feces	
A	Non-cystine	I	gm. 4.71	gm. 0.41	gm. —	gm. 5.12	gm. 3.81	gm. 1.30	gm. 5.11	per cent 74.6	per cent 25.4	gm. +0.01
		II	4.91	0.40	—	5.31	3.53	2.16	5.69	62.0	38.0	-0.38
		Av.	4.81	0.41	—	5.22	3.67	1.73	5.40	68.3	31.7	-0.19
	Cystine	III	5.06	0.38	0.08	5.52	3.19	1.60	4.79	66.6	33.4	+0.73
		IV Av.	5.10 5.08	0.38 0.38	0.08 0.08	5.56 5.54	3.94 3.57	1.85 1.73	5.79 5.29	68.0 67.3	32.0 32.7	-0.23 +0.25
B	Non-cystine	I	3.82	0.41	—	4.23	3.68	1.10	4.78	77.0	23.0	-0.55
		II	3.98	0.40	—	4.38	3.67	1.75	5.42	67.7	32.3	-1.04
		Av.	3.90	0.41	—	4.31	3.68	1.43	5.10	72.4	27.7	-0.80
	Cystine	III	4.10	0.38	0.06	4.54	3.82	1.60	5.42	70.5	29.5	-0.88
		IV Av.	4.13 4.12	0.38 0.38	0.06 0.06	4.57 4.56	3.35 3.59	2.03 1.82	5.38 5.40	66.3 68.4	33.7 31.6	-0.81 -0.85
C	Non-cystine	I	4.41	0.41	—	4.82	3.60	2.71	6.31	57.0	43.0	-1.49
		II	4.59	0.40	—	4.99	3.53	2.25	5.78	61.1	38.9	-0.79
		Av.	4.50	0.41	—	4.91	3.57	2.48	6.05	59.1	41.0	-1.14
	Cystine	III	4.73	0.38	0.07	5.18	3.27	2.06	5.33	61.4	38.6	-0.15
		IV Av.	4.77 4.75	0.38 0.38	0.07 0.07	5.22 5.20	3.05 3.16	2.03 2.05	5.08 5.21	60.1 60.8	39.9 39.3	+0.14 -0.01

TABLE IV  
NITROGEN FINDINGS  
Baked Bean Series

Subject	Expt.	Period	Average daily intake				Average daily output				Balance	
			Beans	Other foods	Cystine	Total	Urine	Feces	Total	Urine		Feces
C	Non-cystine	V	gm. 4.23	gm. 0.35	gm. —	gm. 4.58	gm. 3.49	gm. 1.96	gm. 5.45	per cent 64.0	per cent 36.0	gm. −0.87
		VI	4.36	0.35	—	4.71	3.14	2.10	5.24	59.9	40.1	−0.53
		Av.	4.30	0.35	—	4.65	3.32	2.03	5.35	62.0	38.1	−0.70
	Cystine	VII	4.38	0.37	0.06	4.81	3.30	1.92	5.22	63.2	36.8	−0.41
		VIII	4.33	0.36	0.06	4.75	3.10	1.93	5.03	61.1	38.9	−0.28
		Av.	4.36	0.37	0.06	4.78	3.20	1.93	5.13	62.2	37.9	−0.35
D	Non-cystine	V	6.07	0.35	—	6.42	5.14	2.13	7.27	70.7	29.3	−0.85
		VI	6.24	0.35	—	6.59	4.75	2.41	7.16	66.3	33.7	−0.57
		Av.	6.16	0.35	—	6.51	4.95	2.27	7.22	68.5	31.5	−0.71
	Cystine	VII	6.28	0.37	0.08	6.73	4.79	1.85	6.64	72.2	27.8	+0.09
		VIII	6.18	0.36	0.08	6.62	4.25	1.67	5.92	71.8	28.2	+0.70
		Av.	6.23	0.37	0.08	6.68	4.52	1.76	6.28	72.0	28.0	+0.40
E	Non-cystine	V	4.50	0.35	—	4.85	3.30	1.89	5.19	63.5	36.5	−0.34
		VI	4.63	0.35	—	4.98	3.69	1.45	5.14	71.8	28.2	−0.16
		Av.	4.57	0.35	—	4.92	3.50	1.67	5.17	67.7	32.4	−0.25
	Cystine	VII	4.64	0.37	0.06	5.07	3.49	2.32	5.81	60.1	39.9	−0.74
		VIII	4.59	0.36	0.06	5.01	3.00	1.54	4.54	66.1	33.9	+0.47
		Av.	4.62	0.37	0.06	5.04	3.25	1.93	5.18	63.1	36.9	−0.14

grams per kilogram. When furnished by such foods as milk, bread and milk, and meat, this amount of nitrogen was found by Rose and MacLeod (17) to be sufficient to maintain a generous positive nitrogen balance.

The results of addition of cystine were not striking. However, all subjects responded favorably to it in the Baked Bean Series and two of the three subjects in the Bean Purée Series. The range for the nitrogen balance of the subjects on the non-cystine diet averaged  $-1.14$  to  $-0.19$  grams for the different periods, whereas with cystine it ranged from  $-0.35$  to  $+0.40$  grams. In two cases the average balance became slightly positive; one subject was in approximate equilibrium, and two were somewhat less negative. In the remaining instance, the difference between the non-cystine and cystine periods was negligible, the balance changing only from  $-0.80$  to  $-0.85$  grams.

In three subjects the improvement took place parallel with an increase of nitrogen in the feces which indicated that in these instances, at least, the higher coefficients were not associated with better nitrogen absorption. This work confirmed that of the earlier investigators to the extent that the nitrogen of the beans, prepared as described in these experiments, apparently was not well utilized by the body.

Similarly, Sherman and Winters (18) and Sherman, Winters, and Phillips (19) were unable to secure nitrogen equilibrium on diets in which corn or oats furnished most of the protein of the diet. These results thus appeared to support the generally prevalent idea that vegetable proteins are less adequate, used alone, than those from animal sources. Snyder's statement (24) that navy beans may be eaten in comfort in amounts of 4 ounces or more (dry weight) a day is also confirmed. Further, the findings in this experiment agreed with those of Mitchell (11) and McCollum (7) that navy beans cannot be regarded as a "meat substitute" when used as the chief source of nitrogen in the diet. However, it appeared probable that a diet consisting largely of navy beans as a source of nitrogen may be made adequate with the addition of a sufficient amount of supplementary protein or cystine.

*Calcium Findings.* Tables V and VI show that the subjects were practically all in slightly negative calcium balance throughout the experiment and there was little tendency to acquire equilibrium as the diet was continued. Cystine apparently had no favorable effect on calcium usage since all subjects but one showed an increasingly negative average balance after it was added to the diet. The utilization of calcium seemed to be about equal for the two methods of cooking, the Bean Purée Series having a slight advantage.

TABLE V  
CALCIUM FINDINGS  
Bean Purée Series

Subject	Expt.	Period	Average daily intake			Average daily output				Balance
			Beans	Other foods	Total	Urine	Feces	Total	Urine	Feces
A	Non-cystine	I	gm. 0.287	gm. 0.060	gm. 0.347	gm. 0.042	gm. 0.286	gm. 0.328	per cent 12.9	per cent 87.1
		II	0.314	0.061	0.375	0.053	0.538	0.591	9.0	91.0
		Av.	0.301	0.061	0.361	0.048	0.412	0.460	11.0	89.1
	Cystine	III	0.304	0.061	0.365	0.086	0.460	0.546	16.0	84.0
		IV	0.324	0.058	0.382	0.080	0.468	0.548	14.9	85.1
		Av.	0.314	0.060	0.374	0.083	0.464	0.547	15.5	84.6
B	Non-cystine	I	0.233	0.060	0.293	0.032	0.226	0.258	10.6	89.4
		II	0.254	0.061	0.315	0.041	0.382	0.423	9.6	90.4
		Av.	0.244	0.061	0.304	0.037	0.304	0.341	10.1	89.9
	Cystine	III	0.246	0.061	0.307	0.056	0.362	0.418	13.4	86.6
		IV	0.263	0.058	0.321	0.067	0.442	0.509	13.2	86.8
		Av.	0.255	0.060	0.314	0.062	0.402	0.464	13.3	86.7
C	Non-cystine	I	0.268	0.060	0.328	0.067	0.317	0.384	18.1	81.9
		II	0.293	0.061	0.354	0.085	0.391	0.476	17.8	82.2
		Av.	0.281	0.061	0.341	0.076	0.354	0.430	18.0	82.1
	Cystine	III	0.284	0.061	0.345	0.127	0.351	0.478	26.5	73.5
		IV	0.303	0.058	0.361	0.120	0.334	0.454	26.4	73.6
		Av.	0.294	0.060	0.353	0.124	0.343	0.466	26.5	73.6

TABLE VI  
CALCIUM FINDINGS  
Baked Bean Series

Subject	Expt.	Period	Average daily intake			Average daily output					Balance
			Beans	Other foods	Total	Urine	Feces	Total	Urine	Feces	
C	Non-cystine	V	gm. 0.255	gm. 0.066	gm. 0.321	gm. 0.094	gm. 0.350	gm. 0.444	per cent 21.2	per cent 78.8	gm. -0.123
		VI	0.281	0.065	0.346	0.119	0.367	0.486	24.5	75.5	-0.140
		Av.	0.268	0.066	0.334	0.107	0.359	0.465	22.8	77.2	-0.132
	Cystine	VII	0.254	0.072	0.326	0.104	0.344	0.448	23.2	76.8	-0.122
		VIII	0.256	0.065	0.321	0.101	0.380	0.481	21.0	79.0	-0.160
		Av.	0.255	0.069	0.324	0.103	0.362	0.465	22.1	77.9	-0.141
D	Non-cystine	V	0.366	0.066	0.432	0.068	0.441	0.509	13.4	86.6	-0.077
		VI	0.403	0.065	0.468	0.105	0.530	0.635	16.5	83.5	-0.167
		Av.	0.385	0.066	0.450	0.087	0.486	0.573	15.0	85.0	-0.122
	Cystine	VII	0.364	0.072	0.436	0.095	0.491	0.586	16.2	83.8	-0.150
		VIII	0.367	0.065	0.432	0.106	0.405	0.511	20.7	79.3	-0.079
		Av.	0.366	0.069	0.434	0.101	0.448	0.549	18.4	81.6	-0.115
E	Non-cystine	V	0.271	0.066	0.337	0.029	0.419	0.448	6.5	93.5	-0.111
		VI	0.299	0.065	0.364	0.035	0.387	0.422	8.3	91.7	-0.058
		Av.	0.285	0.066	0.351	0.032	0.403	0.435	7.4	92.6	-0.085
	Cystine	VII	0.270	0.072	0.342	0.030	0.537	0.567	5.3	94.7	-0.225
		VIII	0.272	0.065	0.337	0.037	0.433	0.470	7.9	92.1	-0.133
		Av.	0.271	0.069	0.340	0.034	0.485	0.519	6.6	93.4	-0.179

The urinary calcium was low averaging 15.6 per cent of the entire output. Fecal excretion was correspondingly high.

The results for the bean calcium were scarcely so favorable as those obtained by Rose (15), Blatherwick and Long (2), and other workers with calcium from vegetable sources. They resembled those of Rose and MacLeod (17) where almonds supplied from 85 to 86 per cent of the calcium of the diet. These workers were unable to obtain calcium equilibrium on so large a proportion of almonds until the calcium per kilogram of body weight was increased from 8 to 12 milligrams, whereas with other vegetable foods and with a smaller per cent of almond calcium, from 5.6 to 7.0 milligrams of calcium per kilogram of body weight were ample. The diet used in these experiments furnished from 5.5 to 6.5 milligrams of calcium per kilogram of body weight of which 80 to 85 per cent was obtained from the beans.

While it must be granted that the loss of calcium was not great and that the diet provided a scant margin of safety, there seemed to be little tendency to reach a state of equilibrium on the diet. Apparently this amount of calcium, when supplied largely by beans, was insufficient for maintenance. It is probable that the difference was not in the calcium itself but was due to its association with indigestible material.

*Phosphorus Findings.* The average for each period for each subject as seen in Tables VII and VIII showed a slightly negative phosphorus balance. In four out of six cases the negativity decreased during the cystine periods. When the utilization of the nitrogen improved, with two exceptions, that of the phosphorus improved also. The method of cooking apparently was not an important factor.

In five cases the greater part of the phosphorus was excreted through the feces but the differences were less marked than with calcium. The average loss in the urine was approximately 40 per cent, the remaining 60 per cent being excreted in the feces. In all cases the fecal phosphorus increased during the cystine periods but in only three instances were the differences sufficient to appear significant. Whether this increase of fecal phosphorus meant a poorer digestion of the food, it is impossible to state, but in two out of these three cases there was a parallel decrease in fecal nitrogen indicating better absorption of this element at least.

The average phosphorus intake was low, ranging from an average of 0.551 to 0.769 grams per day or 10.3 to 11.5 milligrams per kilogram of body weight. It is doubtful whether equilibrium could be expected with so small an amount in the food. Certainly the quantities used, when supplied by navy beans, were inadequate to maintain the body in phosphorus equi-





TABLE VIII  
PHOSPHORUS FINDINGS  
Baked Bean Series

Subject	Expt.	Period	Average daily intake			Average daily output					Balance
			Beans	Other foods	Total	Urine	Feces	Total	Urine	Feces	
C	Non-cystine	V	gm. 0.462	gm. 0.079	gm. 0.541	gm. 0.294	gm. 0.380	gm. 0.674	per cent 43.9	per cent 56.1	gm. -0.133
		VI	0.485	0.086	0.571	0.251	0.445	0.696	36.1	63.9	-0.125
		Av.	0.474	0.083	0.556	0.273	0.413	0.685	40.0	60.0	-0.129
	Cystine	VII	0.475	0.090	0.565	0.270	0.404	0.674	40.1	59.9	-0.109
		VIII	0.471	0.092	0.563	0.267	0.430	0.697	38.3	61.7	-0.134
		Av.	0.473	0.091	0.564	0.269	0.417	0.686	39.2	60.8	-0.122
D	Non-cystine	V	0.663	0.079	0.742	0.373	0.476	0.849	43.9	56.1	-0.107
		VI	0.694	0.086	0.780	0.359	0.586	0.945	38.0	62.0	-0.165
		Av.	0.679	0.083	0.761	0.366	0.531	0.897	40.9	59.1	-0.136
	Cystine	VII	0.680	0.090	0.770	0.309	0.539	0.848	36.4	63.6	-0.078
		VIII	0.676	0.092	0.768	0.324	0.453	0.777	41.7	58.3	-0.009
		Av.	0.678	0.091	0.769	0.317	0.496	0.813	39.0	61.0	-0.044
E	Non-cystine	V	0.490	0.079	0.569	0.254	0.405	0.659	38.6	61.4	-0.090
		VI	0.515	0.086	0.601	0.235	0.366	0.601	39.1	60.9	±0.000
		Av.	0.503	0.083	0.585	0.245	0.386	0.630	38.8	61.2	-0.045
	Cystine	VII	0.503	0.090	0.593	0.223	0.553	0.776	28.7	71.3	-0.183
		VIII	0.501	0.092	0.593	0.252	0.428	0.680	37.1	62.9	-0.087
		Av.	0.502	0.091	0.593	0.238	0.491	0.728	32.9	67.1	-0.135

librium although some tendency to improved utilization with continued usage was shown by some subjects. This seemed to be associated with somewhat better retention of nitrogen.

The low urinary excretion of both calcium and phosphorus is to be expected because the diet was low in these elements and high in fiber, both of which are believed to increase excretion of these elements by way of the intestine. However, other factors may also be involved.

It is possible, according to Shohl, Bennett, and Weed (22), that the utilization of both the calcium and phosphorus would have been better had the diet been approximately neutral in reaction. On the other hand, the acids of the fruit juices presumably furnished a favorable medium for absorption of both of these minerals by increasing their solubility.

It also is possible that the large amounts of sugar consumed which, including that of the fruit juices, supplied from 50 to 63 per cent of the total calories, may have affected the absorption of the calcium and phosphorus (Bergeim, 1). Approximately 16 to 20 per cent of the sugar calories was supplied by lactose. The benefit that may have been derived from the latter may have been more or less offset by the depressing effects of the other sugars so it is not believed that this factor could have changed materially the results obtained.

### SUMMARY

A study was made of the utilization of the nitrogen, calcium, and phosphorus of the navy bean. Five adult women served as subjects. The beans were cooked in two ways—as bean purée and baked beans. They were eaten with and without a supplement of cystine in the proportion of 2 per cent of the weight of the calculated protein.

The diet—consisting of beans, purified butter fat, sucrose, lactose, grape juice, and lemon juice—was presumably adequate except for some deficiency of Vitamin D.

The following tendencies were observed:

The trend of the nitrogen balance was increasingly negative while the beans were eaten without the cystine supplement.

Addition of cystine in the above proportions, although showing some irregularities, apparently improved nitrogen retention slightly.

The calcium and phosphorus of navy beans in the amounts used were, as a rule, unable to maintain the balance of these elements in the body. The phosphorus was utilized somewhat better than the calcium and showed a slightly favorable response to addition of cystine.

Improved utilization of the phosphorus in most cases appeared to parallel better retention of nitrogen. No such correlation was noted between calcium and nitrogen or calcium and phosphorus.

The methods of cooking used apparently had little effect upon the usage of the elements studied.

Navy beans seemingly can be eaten in comfort by healthy people in quantities at least as large as 4 ounces, dry weight, daily.

This work on human subjects appears to support the findings of others that navy beans are not entirely satisfactory as the chief source of nitrogen in the diet.

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## THE VALUE OF SOME VEGETABLES IN NUTRITIONAL ANEMIA

By

HAROLD LEVINE, F. B. CULP AND C. B. ANDERSON

*(From the Laboratory of the South Carolina Food Research Commission  
and the Department of Nutrition of the Medical College of  
the State of South Carolina, Charleston.)*

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**T**HE more recent investigations (1 to 4) in the study of nutritional anemia have served to emphasize anew the specificity of copper in supplementing iron in the cure of this form of anemia. During the active controversy as to what mineral elements were concerned in hemoglobin regeneration, the studies were made, for the most part, with solutions of inorganic salts carrying the metals under investigation. As a result, in only a few cases have vegetables been studied in an effort to determine their effectiveness in blood formation. A review of the literature revealed the following studies on the influence of various vegetables when fed in different forms.

Mitchell and Schmidt (5) fed dried spinach to anemic rats and found it effective in the regeneration of hemoglobin. The spinach was fed daily at a level of 1.54 gm. of dried material, furnishing 0.4 mg. of iron. Mitchell and Miller (6) found "a concentrated water extract of dried spinach" effective in blood regeneration in rats. The ash of this extract or an HCl solution of the ash of the extract was also potent in curing anemia.

The Wisconsin workers (7 to 9), in their studies, employed both lettuce ash and an HCl extract of lettuce ash and showed that these sources brought about regeneration of hemoglobin.

Sheets, Frazier and Sulzby (10) studied the effect of feeding dried mustard greens, turnip greens and collards to rats suffering from nutritional anemia. These dried vegetables were found to be potent in hemoglobin regeneration.

From the above resumé of the literature, it is evident that more emphasis has been placed upon the ash than upon the natural food which contains more or less indigestible material. Much needed information is therefore lacking as to the value of various vegetables when consumed in their original or dried state. That the results obtained on the ash of vegetables cannot always be transferred to the natural food is apparent from a study by Bloom (17). This investigator, in a study of the effect of crude fiber on calcium and phosphorus retention in rats, found that the calcium and phosphorus of spinach ash were better utilized than that of either raw or cooked dried spinach.

Since it is possible that the availability of the iron or copper present in the intact or the dried vegetable might be different from that in the

plant ash or in an acid solution of the plant ash, we decided to determine the effectiveness of various dried vegetables when fed to anemic rats in order to ascertain whether or not vegetables can serve as physiologically utilizable sources of these mineral elements. It is believed that the results of such an investigation would be applicable to anemia of nutritional origin in man.

Accordingly the following vegetables—spinach, lettuce, tomato, asparagus, broccoli and turnip greens—were studied for their effectiveness in curing nutritional anemia in the rat. Inasmuch as it was impractical to preserve the same lot of fresh plant tissue for the duration of the experiment, recourse was had to the feeding of the dried ground vegetable. We were particularly interested in turnip greens since this vegetable is a popular salad green in this locality.

#### EXPERIMENTAL

The technic used in the breeding of rats, the production of nutritional anemia, the drawing of blood samples, the determination of hemoglobin, the analysis of vegetables and milk powder for iron and copper, etc., was the same as described in a previous publication (11) from this laboratory with the exceptions indicated below. In place of using reconstituted whole milk powder<sup>1</sup> to produce anemia, the dried whole milk powder and distilled water<sup>2</sup> were fed, since we could find no apparent differences in the behavior of the rats when the two sources of milk were compared. Palmer and Kennedy (12) found average digestibility values of 95.6 per cent and 92.7 per cent for whole milk powder and liquid milk, respectively. Enough milk powder representing the same lot of liquid milk was purchased so as to last throughout the entire experimental period. Upon analysis, the milk powder was found to contain 3.6 p.p.m. of iron and 1.89 p.p.m. of copper. In the present experiments, the rats were placed on a milk régime as soon as the average body weight of the animals in each litter reached 40 gm. (approximately at 21 days of age) instead of 60 gm. as in our previous experiments (11). This change in procedure reduced the time required to produce a marked anemia from a period of 6 to 8 weeks to a period of 4 to 6 weeks. Smythe and Miller (13) in analyzing the iron content of the bodies of rats at different ages found the lowest iron content at 20 days of age. This finding would seem to explain the ability to develop a severe anemia in our 21-day-old rats in a shorter time.

In determining the effectiveness of various vegetables in anemia, two

<sup>1</sup> Klim.

<sup>2</sup> Containing only negligible amounts of iron and copper.

series of curative experiments were carried out. The duration of the curative period in both series was eight weeks.

### *Series I*

In the first series, which was preliminary in nature, a total of 31 rats was used. In this series, the hemoglobin content of the rats' blood was allowed to drop to an average value of approximately 6.0 gm. per 100 cc. of blood before supplements were fed. Hemoglobin determinations were carried out on a group of twenty-eight rats, representing four litters, reared on our stock ration, and approximately the same age (84 to 89 days) as our experimental rats after about 4 weeks on supplements in the curative period. The range in values found was from 14.0 to 16.8 gm. hemoglobin per 100 cc. of blood (indicated as the "normal zone" in Charts I and II) with an average value of 14.8 gm. Mitchell and Miller (4) report a range in values from 15.3 to 16.6 gm. after their colony rats are 7 weeks of age. The blood of our anemic rats at the start of the curative period contained only 40 per cent of the normal content of hemoglobin.

At the start of the curative period, the animals were separated into groups, placed in individual galvanized iron wire cages and fed various supplements as indicated in Table I. In order to insure a sufficiency of vitamins A and D throughout the curative period, each rat was fed one drop of cod liver oil daily in this and in the following series. Krauss (14) found cod liver oil to be without effect in nutritional anemia in rats. Table I and Chart I serve to show clearly the results obtained. As a means of comparison of the effectiveness of the different supplements, 14.0 gm. of hemoglobin per 100 cc. of blood were chosen as an indication of a fair degree of blood regeneration, this value representing the lower limit in the range of normal hemoglobin values for our stock animals. In the present discussion, therefore, this value will be taken to indicate regeneration of hemoglobin on a given supplement. Mitchell and Miller (6) also used this value for comparative purposes in their anemia experiments on rats.

From a study of Table I and Chart 1, the following facts are borne out. The negative control animals on milk alone showed slightly decreasing hemoglobin values as the curative period progressed. The hemoglobin value for this group averaged 7.2 gm. at the start of the curative period for the reason that animals with the highest blood values were purposely placed in this group. The normal control rats on the stock ration maintained an almost constant hemoglobin value throughout the curative period. These same rats had been on this ration throughout the curative period while the remainder of the animals were being rendered anemic on milk. When



TABLE I  
AVERAGE WEEKLY HEMOGLOBIN VALUES\* AT THE SUPPLEMENT WAS ADDED AND AT INTERVALS THEREAFTER, OF ANIMALS RECEIVING VARIOUS AMOUNTS OF IRON AND COPPER IN THE FORM OF DRIED SPINACH OR SOLUTIONS OF INORGANIC SALTS

	Negative controls on milk	Normal controls on stock diet	0.50 Iron mg.	0.20 Copper mg.	0.50 Iron plus 0.050 copper mg.	Dried Spinach		
						Hemoglobin	Average daily spinach intake   gm.	Average daily iron intake mg.
At time† of addition	7.2 (3)‡	14.8 (4)	5.9 (9)	6.2 (3)	6.1 (4)	6.0 (8)	—	—
After addition (weeks)								
1	6.6 (3)	14.7 (4)	7.5 (9)	9.3 (3)	11.1 (4)	9.5 (8)	0.51	0.18
2	6.2 (3)	15.2 (4)	8.4 (8)§	9.6 (3)	13.5 (4)	11.6 (8)	1.08	0.38
3	6.1 (3)	14.9 (4)	10.3 (7)§	9.8 (3)	14.3 (4)	13.1 (8)	1.18	0.43
4	5.9 (3)	14.8 (4)	11.4 (6)§	10.6 (2)§	14.7 (4)	14.1 (8)	1.56	0.56
5	5.8 (3)	15.4 (4)	10.8 (5)§	9.7 (2)	15.0 (4)	14.8 (8)	1.38	0.49
6	5.3 (3)	15.1 (4)	11.7 (5)	9.7 (2)	14.6 (4)	14.9 (8)	1.45	0.52
7	4.8 (3)	14.8 (4)	10.4 (5)	10.3 (2)	14.9 (4)	15.2 (8)	1.23	0.44
8	5.1 (3)	14.8 (4)	11.8 (5)	9.8 (2)	14.8 (4)	15.0 (8)	1.23	0.44
Time (weeks) to reach 14.0 gm. hemoglobin	—	—	—	—	2-3	3-4		
								0.0026
								0.0055
								0.0060
								0.0080
								0.0070
								0.0074
								0.0063
								0.0063

\* Expressed as grams of hemoglobin per 100 cc. of blood.

† After 5-6 weeks of preliminary feeding on dried whole milk and distilled water.

‡ The figures in parentheses refer to the number of rats used in obtaining the average hemoglobin value.

§ Rat died.

|| The average daily dried spinach intake for the 8 weeks experimental period was 1.20 grams, containing 0.43 mg. of Fe and 0.0061 mg. of Cu.

either 0.50 mg. of iron<sup>3</sup> or 0.20 mg. of copper<sup>3</sup> were fed *alone*, a temporary rise in hemoglobin content occurred, followed by an apparent flattening of the hemoglobin curve. Mitchell and Miller (4) who fed 0.1, 0.25, and 0.5 mg. of iron *alone* and Krauss (1) who fed 0.05 mg. of copper *alone*, obtained similar results. Only five of our rats survived at the end of the curative period on 0.50 mg. of iron, death resulting presumably from anemia. Two of the three animals fed 0.20 mg. of copper survived. The ability of

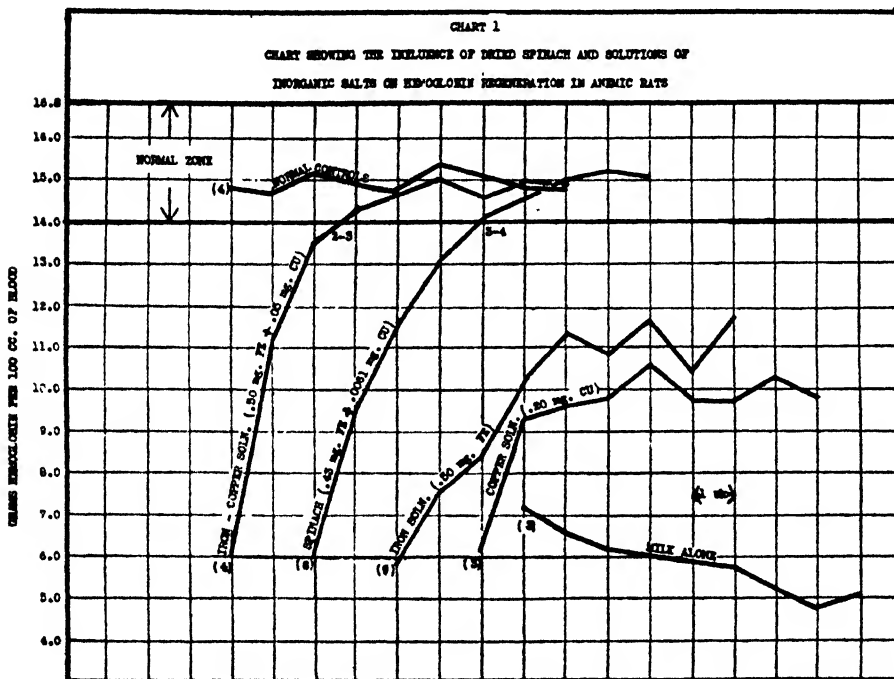


CHART 1. The figures in parentheses at the beginning of each curve refer to the number of animals used in each group. The figures near the top of the curves refer to the time (weeks) required to reach a level of 14.0 gm. hemoglobin per 100 cc. of blood.

either iron *alone* or copper *alone*, to induce partial regeneration of hemoglobin can be ascribed undoubtedly to the presence of small amounts of iron and copper in the milk. When both iron and copper were fed together to the extent of 0.50 mg. of iron and 0.05 mg. of copper, hemoglobin regeneration was speedily effected in 2 to 3 weeks, thereby again confirming the marked supplementary action of copper.

\* The iron ( $\text{FeCl}_3$ ) solution used in these tests was freed from copper by means of  $\text{H}_2\text{S}$  precipitation and, on analysis, was found to introduce only negligible amounts of copper as a contaminant, i.e., 0.000035 mg. of copper daily. Likewise, the copper ( $\text{CuSO}_4$ ) solution was found to contain only minute traces of iron.

Before planning an extensive experimental series in which various dried vegetables were to be fed, it was first desirable to ascertain whether anemic rats would consume such dried plant material and also whether the amounts consumed would insure a sufficient intake of iron so that a comparison of the effectiveness of different vegetables could be made possible. Accordingly, dried ground spinach was fed *ad libitum* to a group of eight rats. The iron and copper content of the spinach are given in Table II. Table I shows that during the first week of the curative period, the animals consumed an average of only 0.51 gm. of the dried vegetable, yielding an average daily intake of 0.18 mg. of iron and 0.0026 mg. of copper. Subsequently, however, the intake of spinach was increased to an average daily intake of over 1.0 gm. The average daily intake of the eight animals for the eight weeks curative period was 1.20 gm., yielding an iron intake of 0.43 mg. and a copper intake of 0.0061 mg. Hemoglobin regeneration was effected in 3 to 4 weeks, thereby indicating that this vegetable when fed *ad libitum* in the dried, ground form can serve as a potent source of the metals concerned in blood formation.

### Series II

Our experience with the feeding of dried ground spinach *ad libitum* in Series I demonstrated that the anemic rat would ingest sufficient amounts of this plant material to effect rapid regeneration of hemoglobin. With this information at hand, we decided to investigate the potency of some other vegetables and also to make a comparison of several vegetables when fed at the same level of iron. Table II gives the iron and copper con-

TABLE II  
SHOWING THE IRON AND COPPER CONTENT OF SOME VEGETABLES

Vegetable	Dry matter (%)	Dry Basis (p.p.m.)		Fresh basis (p.p.m.)	
		Iron	Copper	Iron	Copper
Asparagus	6.7	253	11.0	17.0	0.74
Broccoli	10.5	200	2.0	21.0	0.21
Lettuce	6.5	364	5.3	23.7	0.35
Spinach	9.1	356	5.1	32.4	0.46
Turnip greens	11.0	300	12.5	33.0	1.38
Tomato	5.0	150	14.2	7.5	0.71

tent of the six vegetables used in this investigation, while Table III shows the amounts of iron and copper contained in the daily vegetable supplements.

The vegetables fed were grown in various localities of South Carolina. In our laboratory, Remington and Shiver (15) in a study of eighteen of the more commonly used vegetables grown in South Carolina, found considerable variation in the iron, copper and manganese content of each vegetable. It is to be emphasized, therefore, that standard values for iron, copper or manganese content cannot be assigned to such plant materials. The samples used in the present investigation represent vegetables having an iron and copper content close to the lower limit of the range in values reported by Remington and Shiver for these vegetables.

As indicated in Table III, a lettuce and tomato mixture was also fed. This mixture was prepared in such proportions that each vegetable furnished one-half of the total amount of iron present in the daily supplement. We were interested in feeding this mixture for the reason that this combination of vegetables is quite often used as a salad. Since turnip greens represent a rather favorite salad in the south, we were interested in determining the effectiveness of this vegetable.

TABLE III  
SHOWING THE AMOUNTS OF IRON AND COPPER CONTAINED IN THE  
DAILY DRIED VEGETABLE SUPPLEMENTS

Vegetable		Amount fed		Equivalent on a fresh basis		Iron		Copper	
		gm.		gm.		mg.		mg.	
Asparagus		0.79		11.8		0.200		0.0087	
Broccoli		1.00		9.5		0.200		0.0020	
Lettuce		0.55		8.5		0.200		0.0029	
Lettuce and tomato mixture	Lettuce	0.28	0.95	4.4	17.8	0.100	0.200	0.0015	0.0110
	Tomato	0.67		13.4		0.100		0.0095	
Turnip greens		1.43		15.7		0.425		0.0179	
Spinach		0.56		6.1		0.200		0.0029	

As indicated in Table III, asparagus, broccoli, lettuce, lettuce and tomato mixture and spinach were all fed at a level furnishing 0.20 mg. of iron daily but different amounts of copper. This low level of iron was chosen instead of a higher level for the reason that we wished to deal with

TABLE IV  
AVERAGE WEEKLY HEMOGLOBIN VALUES\* AT TIME SUPPLEMENT WAS ADDED AND AT INTERVALS THEREAFTER, OF ANIMALS RECEIVING  
IRON AND COPPER IN THE FORM OF DRIED VEGETABLES OR SOLUTIONS OF INORGANIC SALTS

	Negative controls on milk	Lettuce and tomato mixture (0.95 gm.)	Asparagus (0.79 gm.)	Lettuce (0.55 gm.)	Spinach (0.56 gm.)	Broccoli (1.00 gm.)	Turnip greens (1.43 gm.)	Iron solution	Iron plus copper solution
Fe in supplement (mg.)	—	0.20	0.20	0.20	0.20	0.20	0.425	0.25	0.25
Cu in supplement (mg.)	—	0.0110	0.0087	0.0029	0.0029	0.0020	0.0179	0.00	0.0170
At time† of addition	5.0 (6)†	4.4 (5)	4.5 (6)	4.6 (12)	4.3 (13)	4.8 (6)	4.8 (4)	4.2 (6)	4.8 (3)
After addition (weeks)									
1	5.1 (6)	7.6 (5)	8.2 (6)	7.9 (12)	7.0 (13)	6.1 (6)	7.8 (4)	5.6 (6)	7.8 (3)
2	4.7 (6)	9.2 (5)	10.5 (6)	9.1 (12)	9.0 (13)	7.5 (6)	9.4 (4)	7.3 (6)	10.1 (3)
3	4.7 (6)	12.1 (5)	11.9 (6)	11.8 (12)	11.4 (13)	9.7 (6)	13.5 (4)	8.4 (6)	13.0 (3)
4	4.9 (6)	13.2 (5)	12.8 (6)	12.3 (12)	12.6 (13)	10.1 (6)	14.2 (4)	9.1 (6)	13.6 (3)
5	4.7 (6)	14.0 (5)	13.4 (6)	13.0 (12)	12.8 (13)	11.4 (6)	14.8 (4)	10.2 (6)	14.3 (3)
6	4.6 (6)	14.7 (5)	14.2 (6)	13.6 (12)	13.5 (13)	13.0 (6)	15.1 (4)	11.3 (6)	14.9 (3)
7	4.7 (5)§	15.5 (5)	14.6 (6)	14.3 (12)	14.4 (13)	13.7 (6)	15.4 (4)	11.7 (6)	15.3 (3)
8	4.6 (5)	15.5 (5)	15.1 (6)	15.3 (12)	15.5 (13)	15.1 (6)	15.6 (4)	11.8 (6)	16.2 (3)
Time (weeks) to reach 14.0 gm. hemoglobin.	—	4-5	5-6	6-7	6-7	7-8	3-4	—	4-5

\* Expressed as grams of hemoglobin per 100 cc. of blood.

† After 4 to 6 weeks of preliminary feeding on dried whole milk and distilled water.

‡ The figures in parentheses refer to the number of rats used in obtaining the average hemoglobin value.

§ Rat died.

a relatively slow rate of hemoglobin regeneration thereby allowing differences in the rate of regeneration of the different vegetables to appear. Mitchell and Miller (4) recommend a level of 0.25 mg. of iron when comparisons are to be made. Turnip greens were fed at a level yielding daily 0.425 mg. of iron and 0.0179 mg. of copper in order to ascertain whether this vegetable would bring about blood formation as rapidly as the spinach did in Series I. Table IV and Chart 2 show the results obtained in this

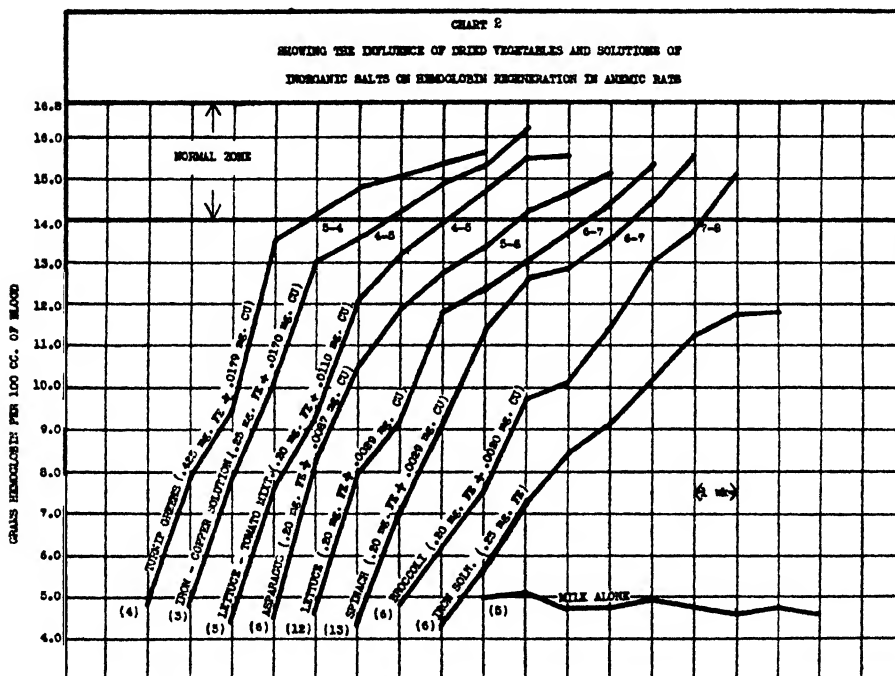


CHART 2. The figures in parentheses at the beginning of each curve refer to the number of animals used in each group. The figures near the top of the curves refer to the time (weeks) required to reach a level of 14.0 gm. hemoglobin per 100 cc. of blood.

series. As indicated in the table, beside the vegetable-fed rats, three other groups receiving milk, 0.25 mg. of iron *alone* and 0.25 mg. of iron plus 0.0170 mg. of copper respectively, were included.

In this series, the average hemoglobin content of the rats' blood was reduced to approximately 4.5 gm. per 100 cc. of blood, i.e., 30 per cent normal, before supplements were fed. This value was 10 per cent lower than that in Series I and therefore represents a more marked anemia. The importance of a low initial hemoglobin in studies on nutritional anemia has been pointed out by Mitchell and Miller (4).

From a study of Table IV and Chart 2, it is evident that all the vegetables studied exhibited the capacity to regenerate hemoglobin in animals suffering from nutritional anemia. In comparing the different vegetables, the data show that the vegetables brought about hemoglobin regeneration at rates depending on the copper content. Thus, lettuce and tomato mixture, asparagus, lettuce, spinach, and broccoli—all fed daily at a level affording 0.20 mg. of iron but different amounts of copper—permitted hemoglobin regeneration in 4 to 5, 5 to 6, 6 to 7, 6 to 7, and 7 to 8 weeks, respectively, in the order of decreasing copper intake. Turnip greens effected regeneration in 3 to 4 weeks, comparing favorably, therefore, with the results obtained with spinach in Series I.

In connection with these findings, it is to be emphasized that due to the variations in iron and copper known to exist in vegetables, another set of the same vegetables when fed on the same basis would probably yield results giving a different order of anti-anemic potency.

The present results, therefore, serve to emphasize the importance of the above-mentioned vegetables in the diet since they furnish both iron and copper in a form available to the body.

When 0.25 mg. of iron *alone* was fed, a result similar to that obtained with 0.50 mg. of iron in Series I was obtained. The supplement furnishing 0.25 mg. of iron and 0.0170 mg. of copper effected blood formation in 4 to 5 weeks. As indicated in Chart 2, the hemoglobin values for all the groups except the negative controls and the group of rats fed 0.25 mg. of iron, entered the "normal zone" at some time during the eight-weeks curative period. In confirmation of the results obtained by other investigators, the results of the present two series of experiments emphasize again the importance of both iron and copper in blood formation.

### Discussion

The results obtained in the feeding of dried vegetables serve to demonstrate that such foods are of great importance in the maintenance of normal blood formation. It is apparent that the vegetables studied furnish both iron and copper in a form available to the body for the formation of hemoglobin. The extent to which vegetables contribute to the iron and copper content of sixteen different menus given by Rose (18) for children, adolescents, men and women, has been studied by Hodges and Peterson (19). These workers found that vegetables and cereals are the chief contributors of iron to the diet and supply about equal proportions of this element, i e., about 21 to 24 per cent of the total iron. In respect to copper, vegetables with an average contribution of 19 per cent rank second to cereals supplying 35 per cent.

In confirmation of the results of Waddell and co-workers (16), the results of the present experiments bring out the importance of the small amount of copper which can render iron effective. In our study, broccoli furnished the lowest amount of copper, i.e., 0.0020 mg. daily, which, in conjunction with 0.20 mg. of iron, effected blood regeneration in 7 to 8 weeks. The Wisconsin investigators (16), in their studies involving solutions of inorganic salts, found as little as 0.0025 mg. of copper to possess the ability to induce hemoglobin formation in anemic rats. In our experiments, the group of rats fed the highest amount of copper experienced the most rapid blood regeneration. Thus, in the vegetable-fed groups receiving 0.20 mg. of iron, the lettuce and tomato combination which furnished the largest amount of copper, i.e., 0.0110 mg. daily, effected hemoglobin regeneration in the shortest time, i.e., 4 to 5 weeks.

In Series II, whereas lettuce alone permitted regeneration of blood in 6 to 7 weeks, the lettuce and tomato combination effected recovery in only 4 to 5 weeks. This difference in the rate of regeneration can be ascribed to the higher copper content of the tomato. Remington and Shiver (15) in a study of eighteen of the more commonly used vegetables, found the tomato to contain the largest amount of copper. Since the tomato is much lower in iron than lettuce, and vice-versa much higher in copper, it would appear that these two vegetables supplement each other to make a combination much more effective than either vegetable alone. From a nutritional standpoint, therefore, a lettuce and tomato salad is to be highly recommended.

### Summary

The influence of various dried vegetables in the cure of nutritional anemia was studied.

Dried spinach when fed *ad libitum* and yielding an average daily intake of 0.43 mg. of iron and 0.0061 mg. of copper, effected hemoglobin regeneration in 3 to 4 weeks.

Lettuce plus tomato mixture, asparagus, lettuce, spinach, and broccoli—all fed at a level affording 0.20 mg. of iron but different amounts of copper—permitted hemoglobin regeneration in 4 to 5, 5 to 6, 6 to 7, 6 to 7, and 7 to 8 weeks, respectively, in the order of decreasing copper intake. Since variations in the iron and copper content of these vegetables are known to exist, it is to be borne in mind that a different order of anti-anemic potency would probably result from another set of the same vegetables. Turnip greens fed at a level furnishing 0.425 mg. of iron and 0.0179 mg. of copper brought about rapid regeneration in 3 to 4 weeks.



When fed at the same level of iron, a lettuce and tomato combination was found more effective than lettuce alone.

The above vegetables are, therefore, important sources of the minerals concerned in normal blood formation.

Iron *alone* or copper *alone*, when fed in the form of inorganic salt solutions, permitted only partial blood regeneration, whereas solutions containing both iron and copper effected rapid recovery of hemoglobin.

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# THE RELATIVE QUANTITIES OF THE HEAT-STABLE AND HEAT-LABILE FRACTIONS OF VITAMIN B IN RAW AND EVAPORATED MILK\*

By

LEO T. SAMUELS AND FRED. C. KOCH

*(From the Department of Physiological Chemistry and  
Pharmacology of the University of Chicago)*

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## INTRODUCTION

**D**URING the last five years the distinction of at least two factors in vitamin B has been well established (10, 12, 29, 5). As early as 1920 Emmett and Luros (10) showed that one factor could be destroyed by autoclaving at 120° C. for two hours or more. Funk and Dubin (12) showed differences in adsorbability on fuller's earth, and Chick and Roscoe (5) showed differences in solubility in alcohol. The heat-labile substance, more soluble in alcohol, cured polyneuritis in pigeons. The heat-stable fraction was thought by Goldberger (13) to relieve pellagra.

Of particular interest is the evidence which Evans (11), Sure (30), Macy (21), Hartwell (15), Daniels (6), and others have given of the dependence of the mother's milk upon the vitamin B content of her diet, and of a large dissipation of the vitamin in her body, with consequent interference with the adequacy of her milk normally to support the young. Macy (21, 24) has shown by direct growth experiments that human milk and cow's milk are both relatively low in this vitamin, and she, as well as Hunt and Krauss (17), Daniels (6, 7), and Hartwell (15) give evidence that the antineuritic fraction is particularly low in cow's milk. Blossom (2) showed that the addition of yeast to infant dietaries increased the rate of growth 60 to 100 per cent. It then may well be that vitamin B is below the optimum level in many infant dietaries.

With the increasing number of infants who are artificially fed and the greater use of evaporated milk for this purpose, as advocated by Marriott (22) and Brenneman (4), together with the interest aroused in the use of milk for the treatment of pellagra, the relative amounts of the factors of the B complex in normal fresh cow's milk and evaporated milk become an important problem. This has been attacked by a number of investigators with confusing results, due to differences in method and in the degree of accuracy obtained. Dutcher, Francis and Combs (9), using direct feed-

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\* This work was done during the tenure of a fellowship from the Evaporated Milk Association.

ing experiments and preparing the evaporated milks in the laboratory could find no difference between raw and heat-treated milks. However, the amounts of milk fed were low and the experiments were discontinued at a time when differences seemed to be appearing in the growth curves. Johnson and Norton (18) reached the same conclusions in regard to the two types of milk, as did Daniels and Loughlin (8) in some early studies but recently Hartwell (15), and Daniels and co-workers (6, 7), using Hartwell's lactation method, have found great differences. Sure (33) has criticized this work on the basis of failure to deplete the reserves of the mothers, and inadequacy of the basal ration in minerals. It was considered worth while therefore to attempt a quantitative comparison in raw and evaporated milk of the factors of the B complex which could be clearly distinguished.

#### EXPERIMENTAL

The experimental work can conveniently be divided into three parts:

A. The separation of the two factors by fractionation of the milk solids and confirmation that the limiting factor is the heat-labile fraction.

B. Quantitative estimation of the relative amounts of the heat-labile fraction in raw and evaporated milks.

C. Quantitative estimation of the heat-stable fraction in raw and evaporated milks.

The rats used for the growth experiments were from stock litters weaned on the twenty-first day at a weight of 40 to 50 gm. At that time they were transferred to cages with one-half inch mesh wire false bottoms, which were thoroughly cleaned and sterilized once a week. They then received the basal ration plus water *ad libitum* until they ceased to gain weight, when they were divided and fed the substances to be tested in addition. Where comparisons were to be made, care was taken to distribute comparable litter mates of the same sex among the various groups. All substances fed in addition to the basal ration occupied separate containers. Thus, consumption could be accurately determined. If the substances were fed in a moist powdered, or liquid form there was little if any loss.

The basal ration consisted of:

Casein, purified	20 parts
Starch, Harris, B-free	64 "
Butterfat, purified	8 "
Agar agar, Merck	4 "
Salt mixture, McCollum	4 "
Cod liver oil, Squibb	2 "

In our later experiments one drop of cold liver oil was given with the test substance to insure adequate intake of the fat-soluble vitamins.

The casein was prepared from commercial casein by dissolving in dilute ammonium hydroxide and precipitating with dilute acetic acid according to a modification of the method of Bosworth and Van Slyke (3). The solution and precipitation were repeated four times. The precipitated casein was then held for four days in 0.01 per cent acetic acid, the acid being changed at least once a day. Next the casein was treated for 24-hour periods with three portions of ethyl alcohol, and finally extracted with two portions of ether. The resultant white, friable, tasteless product was dried in a current of warm air and ground to a fine powder.

The butterfat was purified by melting creamery butter on the water bath until the fat layer had thoroughly separated, and then filtering this layer through filter paper in a hot water funnel.

We used autoclaved yeast as the source of the heat-stable fraction and an antineuritic extract prepared according to the method of Kinnersley and Peters (19, 20) for the heat-labile fraction in connection with the milk studies.

The yeast was prepared by drying Fleischmann's starch-free yeast in a current of warm air after crumbling the cakes. The dry yeast was then ground to a powder. The powder was spread in thin layers in tin pans and autoclaved at a steam pressure of 15 lbs. for four hours. It was then dried again in warm air and ground to a powder. When this was fed in quantities of 1.0 gm. a day as an addition to the diet, rats which had come to a standstill on the basal ration gained weight for a brief period and then rapidly declined, death occurring in 23 to 26 days. Toward the end of their days all showed convulsions. Typical curves are shown in Chart 3.

For the antineuritic extract the Kinnersley and Peters procedure was followed in most cases as far as the N/10 hydrochloric acid extract of the norite charcoal. It was preserved by addition of alcohol to give 15 to 20 per cent concentration. To avoid the use of mercury the modification (20) was used in which a norite adsorption at pH 2.5 was substituted for the mercuric sulfate precipitation, except in two extracts, where the regular procedure was followed. With this extract alone as the source of this vitamin, the rats failed to gain but maintained a level of weight for three to four weeks after which they gradually declined. See also Chart 3.

When the autoclaved yeast was fed with the antineuritic extract, however, the rats grew normally to adult size. The two products must then supply all of the vitamin B complex.

*A. Separation of the two factors by fractionation of the milk solids and confirmation that the limiting factor is the heat-labile antineuritic fraction:*

In the first experiment an attempt was made to separate the two water-

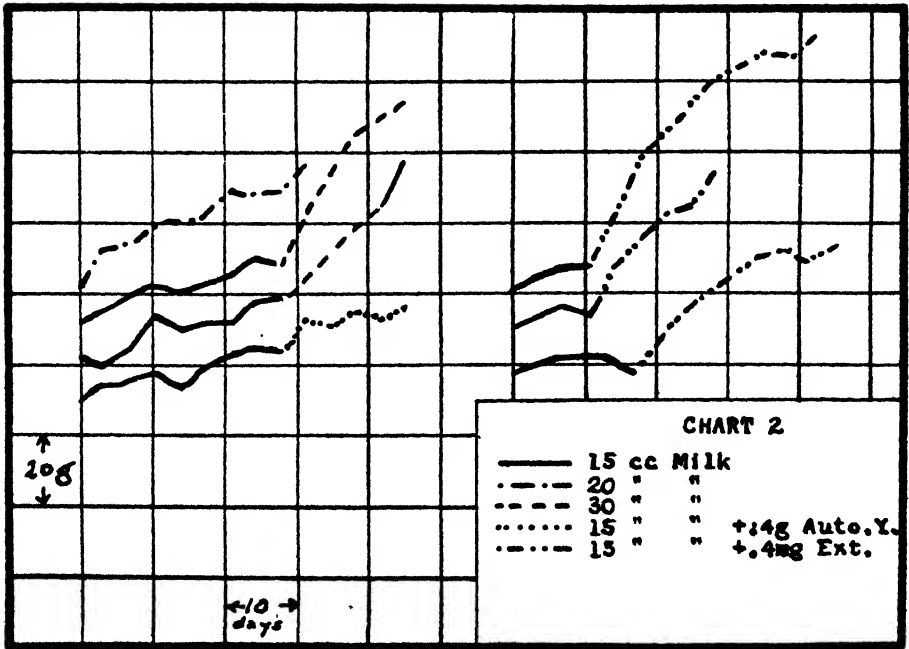
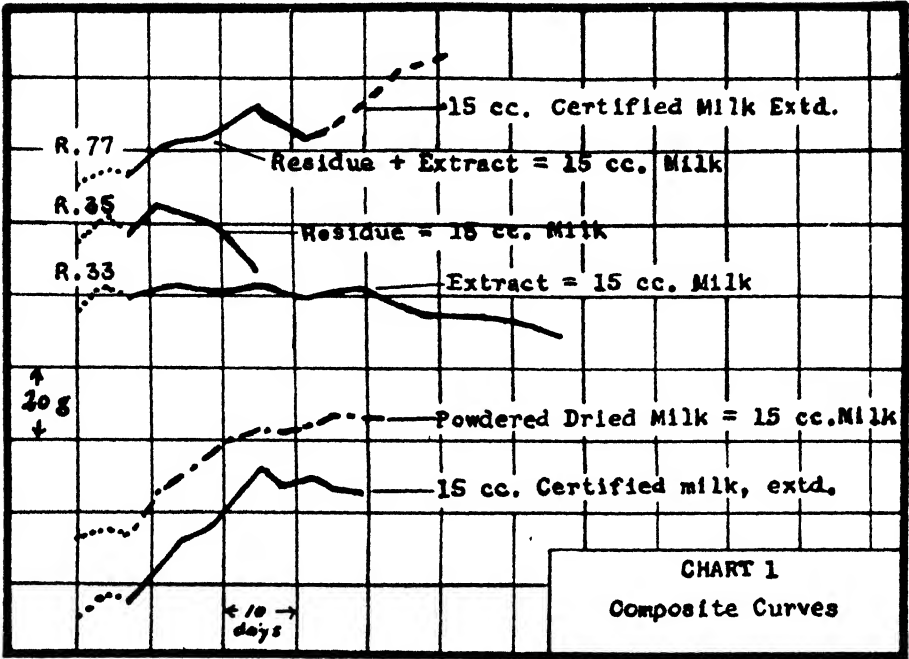
soluble B fractions on the basis of their difference in solubility in alcohol, as shown by Salmon (26), Chick and Roscoe (5), and Sherman (27). Sixteen quarts of certified milk were mixed in a large container after the cream had been drawn off. This milk was then extracted three times with equal volumes of ether previously washed with water. Five liters of the ether-extracted milk were placed in a large flask and aerated until the ether was completely removed. This was then filled into 150 cc. Erlenmeyer flasks and stored at a temperature of  $-4$  deg. C. This served as a control on the ether extraction.

After the lipoids had been removed so as not to interfere with the extraction, the balance of the milk was placed in shallow pans and evaporated to dryness in a current of warm air, ground to a powder and 13/16 of the total milk solids extracted with 85 per cent alcohol. The extract supposedly contained the heat-labile fraction and the residue contained the heat-stable part. As a source of fresh milk for comparison, unpasteurized milk was used.

Chart 1 illustrates the results obtained in this experiment. The first curve shows that the rats made some growth when the extract and residue were combined in amounts equivalent to 15 cc. of the ether-extracted milk but soon leveled off. When 15 cc. of the ether-extracted milk were fed instead, the rats again started to grow, although at a sub-normal rate. There was obviously a loss between the ether extraction and the final alcohol extract and residue. Was this due to the drying or the alcohol extraction? The two curves at the bottom of the chart show that the dried, ether-extracted-milk powder was just as efficient a source of the complex as the original, fresh certified milk. No destruction had occurred in drying; the loss took place during the alcohol extraction. This also showed that 15 cc. of milk could not adequately supply the two factors.

The second and third curves on the chart show that the extraction, however, effected a distinct separation. The second curve duplicates the control curves with autoclaved yeast. The rats did not develop polyneuritis as early as is usual with the rats on the autoclaved yeast, however, which would seem to indicate a slight trace of the antineuritic substance still present. The third curve, in which the extract alone was fed in addition to the basal, is a typical curve as given in the presence of the heat-labile fraction only. Evidently the alcohol extracted practically all of the heat-labile, antineuritic substance without extracting much of the heat-stable fraction. This gives positive evidence of the presence of the two fractions in milk, but the loss in the process prevents its use for quantitative work.

The second series of studies was devoted to determining which was the



limiting factor in milk. Chart 2 illustrates these findings. Rats which had ceased to grow rapidly at a subnormal level on 15 cc. of milk did not improve when autoclaved yeast was added, but did resume normal growth when the Kinnersley and Peters antineuritic extract was added or when the milk level was doubled. Twenty cubic centimeters of milk did not give normal growth however. The limiting factor is, then, the antineuritic, heat-labile fraction as reported by Macy (24) and Hunt and Krauss (17), and the amount of cow's milk necessary to furnish adequate vitamin must lie between 20 and 30 cc. Attention is also called to the tendency of the rats on 15 cc. of milk, plus the antineuritic extract, to level off after a time. This will be discussed later in connection with observations on the heat-stable fraction.

*B. Measurement of the antineuritic heat-labile fraction in milk.*

Having determined the approximate levels necessary for normal growth we now planned more quantitative studies. Sherman's method (28), while having the advantage of shorter time, would not give us direct information as to the level required for normal development to the adult stage. Two other growth methods were used and compared with the lactation method of Sure (31, 32).

In all quantitative growth studies litters were selected which had a sufficient number of similar individuals of the same sex to permit distribution through a majority of the groups in the experiment. Particular care was paid to groups on equivalent amounts of raw and evaporated milk, as nearly as possible exact duplicates being distributed between the two groups. Sexes were distributed in the same ratio in all groups of a series. Thus we found it possible to use groups of six rats and obtain reproducible curves. However, six rats are not enough, if comparisons are to be made between different groups at different times. The rats in a group were not all started on the same day but generally over three different weight periods three days apart. Thus we found that we offset the variables in weight due to transitory changes in environment since the same relative gain in weight of the different rats would span different periods. The composite curves then become more smooth and readable.

Since the diet of the lactating animal has been shown to affect the vitamin content of the milk (15, 30), the only way to form an adequate idea of the effect of commercial evaporation was to compare the same milk before and after treatment.<sup>1</sup> Aliquots of the raw milk were drawn from the

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<sup>1</sup> Through the courtesy of the Carnation Milk Products Co. we were able to obtain these milk samples under ideal conditions.

thoroughly stirred receiving tanks, stored in ice, thoroughly mixed, sealed in tins and shipped immediately, packed in ice, to the university where they were stored at a temperature of  $-4^{\circ}\text{C}$ . Samples of the evaporated milk produced from this raw milk were also shipped and stored at a temperature of  $5^{\circ}\text{C}$ . These two samples of the same milk before and after treatment were used for the main series in each experiment. As controls, samples of certified milk and evaporated milk, purchased on the open market, were run at a level of 15 cc. This level was chosen because it shows the greatest variation in growth per unit difference in vitamin intake.

The comparison between fresh certified milk and the frozen raw milk from the company supplying it shows that six months' storage at  $-4^{\circ}\text{C}$ . had not affected the antineuritic content sufficiently to render it inferior to the fresh product (Chart 4). Evidently the antineuritic factor is quite stable under these conditions.

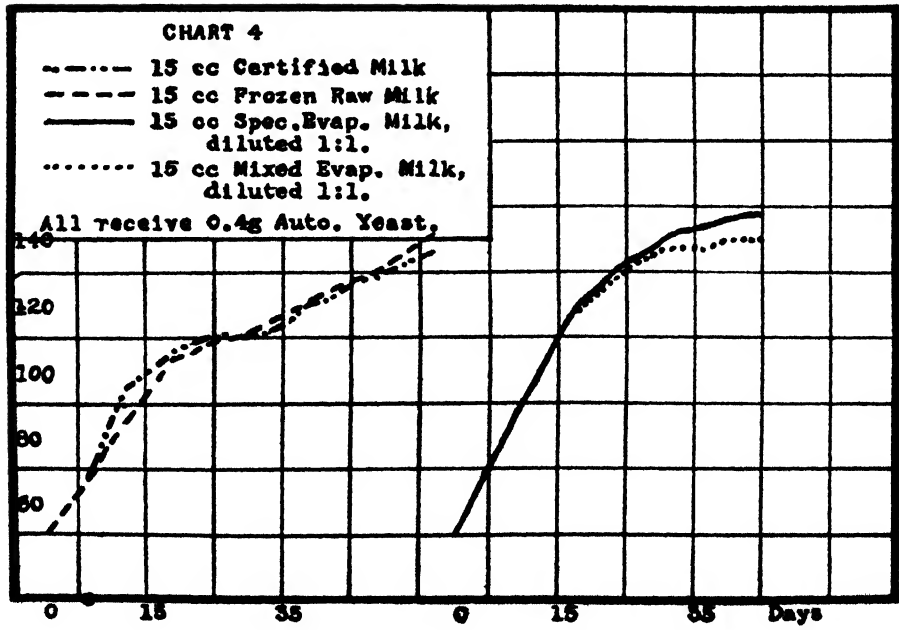
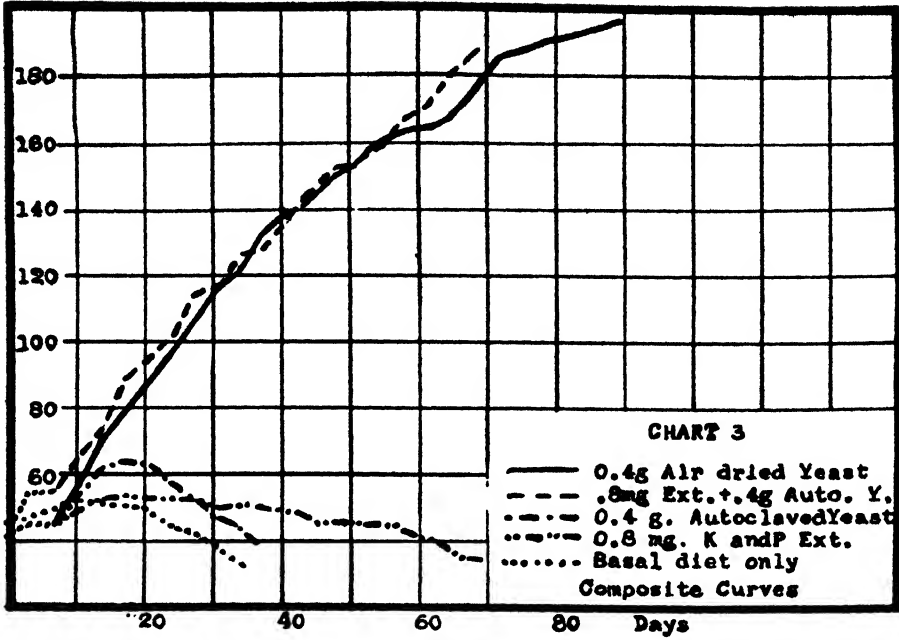
The other set of curves in Chart 4 demonstrates that the sample of evaporated milk used in these experiments was a fair representative of the evaporated milk on the market, since it gave the same or very slightly better growth than the mixed evaporated milk made up of equal quantities of two milks under producers' labels and two milks under distributors' labels.

The method used in the first experiment, comparing the same milk before and after commercial evaporation, consisted simply in feeding a series of different amounts of milk to rats receiving the basal ration plus 0.5 gm. of autoclaved yeast daily. The evaporated milks were diluted with an equal quantity of distilled water both because this is the usual way evaporated milk is used and because analysis showed that this gave milk of very nearly the original composition. This is shown by the following analyses on the special raw milk and the evaporated milk made therefrom:

	Raw %	Dilute Evap. %
Lactose . . . . .	5.10	5.05
Protein . . . . .	3.48	3.50
Calcium . . . . .	0.129	0.130
Phosphorus . . . . .	0.093	0.100
Fat . . . . .	3.81	Undet.

On the basis of this analysis, the ratio of total calories to protein calories is 4.63 while in the basal ration the ratio is 5.14. To adjust the composition 0.018 gm. of starch and 0.007 gm. of agar agar were added per cubic centimeter of milk. The results are shown in the composite curves in Chart 5. The amounts of evaporated milk noted on all the charts are





amounts of evaporated milk diluted 1:1 with water. The curves for the raw milk in this series are slightly above those for the same quantity of

diluted evaporated milk until the quantities of both are high enough to give normal growth. Twenty-five cubic centimeters of raw milk have given better growth than 25 cc. of evaporated milk, or 20 cc. of either, but no better than 30 cc. of evaporated. These both give about the same growth as the normal controls, so that the ratio of the antineuritic vitamin must be inversely as 25 cc. raw to 30 cc. dilute evaporated. That the method is sensitive to the differences we are measuring, is shown by the rapid break when the amounts in two groups were lowered and the way in which one group again crosses the lower levels when returned to the higher quantities.

TABLE I

Group	Milk fed	Antineuritic extract cc.	Frac.* normal	Milk** equivalent	Av. Wt. 50 days on diet.
90	None	0.0	0	—	dead
91	15 cc. dil. evap.	0.0	0	15 cc. dil. evap.	122.5
92	" " "	0.2	$\frac{1}{2}$	22.5 " " "	155.4
93	" " "	0.4	$\frac{1}{2}$	30 " " "	186.7
94	" " "	0.5	$\frac{1}{2}$	33.75 " " "	191.0
97	15 cc. raw	0.0	0	15 cc. raw	134.2
98	" "	0.2	$\frac{1}{2}$	21 " "	174.8
99	" "	0.3	$\frac{1}{2}$	24 " "	185.9
100	" "	0.4	$\frac{1}{2}$	27 " "	188.0
103	15 cc. certified	0.0	0	15 —	132.0
96	None	0.8	1	—	187.5
102	Dried yeast 0.5 g.				193.0

\* This column gives the fraction of the standard dose of the antineuritic fraction which was fed.

\*\* This column gives the amount of milk to which this diet is equivalent on the basis of Group 99 as the minimum normal for raw milk and Group 93 as the minimum normal for dilute evaporated milk. Thus, since 15 cc. of raw milk are equivalent to  $\frac{1}{2}$  of the normal,  $\frac{1}{2}$  of the normal dose of antineuritic extract must equal 6 cc. of milk, and the diet of Group 98 must be equivalent to  $15+6$  cc. = 21 cc.

The above method, while giving fair results, is open to criticism since while one may adjust the milks to resemble the basal ration on a gross basis, it is still true that with varying quantities of milk one also varies the amounts of lactalbumin, lactoglobulin, lactose, etc. The best method of measuring the vitamin value of a substance would be one in which only the vitamin itself would be varied. The second method was devised to approach this condition. All groups were fed the same subnormal level of milk, 15 cc. lying in the range of widest weight variation per unit vitamin. To this were then added varying quantities of an accurately standardized anti-

neuritic extract prepared as previously mentioned. Of this preparation 0.858 mg. daily gave normal growth up to at least 200 gm. body weight. Since the highest level of this fed to any milk group was  $\frac{5}{8}$  of the normal (0.536 mg.), this was the widest variation in intake aside from the basal ration, thus lowering this possible error considerably. Six rats were used in each group, four males and two females. Great care was taken to have uniform general distribution. The results are given in Chart 6 and Table I. The curves show even better results than the previous experiment. According to this series 24 cc. of raw milk are equivalent to 30 cc. of the diluted evaporated milk and will give normal growth. The ratio is then between 4:5 and 5:6. In other words approximately  $\frac{1}{5}$  of the antineuritic heat-labile fraction is destroyed in the process of commercial evaporation.

In our last series of experiments we used a lactation method, since the experiments of Hartwell and Daniels by Hartwell's method had indicated wider differences between raw and evaporated milks. However, to avoid the errors in Hartwell's diet pointed out by Sure (33), we decided upon a method very similar to that of the latter workers (32). Females were transferred upon the day of birth of their litters to the vitamin B-free basal ration plus an allowance of 1.0 gm. of autoclaved yeast daily. The litters were reduced to six within three days after birth. Since the young at this age would fall through the false bottoms of our cages, they were placed on pure white crepe paper in the bottom of the cage, but were transferred to the false bottoms as soon as they grew sufficiently. This was

TABLE II  
LACTATION EXPERIMENTS WITH EVAPORATED AND RAW MILK

No.	No. in litter	Days on basal	Av. wt. at transfer	Days on milk	No. weaned	Ave. wt. weaned	Condition
40 cc. raw milk:							
362	6	14	12.4	22	0		Squealing fits, convulsions
250	6	16	24.0	16	6	41.5	
337	6	16	20.3	24	1	37	Rats died in severe convulsions
50 cc. raw milk:							
243	6	16	22.4	15	6	42.3	Normal
245	6	16	17.8	17	6	40.7	Normal
339*	6	3	5.4	23	6	39.8	Normal

TABLE II (cont'd)

## 60 cc. raw milk:

358	6	15	16.6	18	6	40.8	Normal
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## 70 cc. raw milk:

254	6	17	24.6	9	6	40.3	Normal
328	6	16	15.9	13	6	39.6	Normal
296†	6	14	19.3	20	6	33.3	2 rats had running fits

## 25 cc. undiluted evaporated milk:

293‡	6	15	18.5	17	6	37.0	2 rats had convulsions, very weak
170	6	11	13.6	19	5	40.7	1 rat died in convulsions
312	6	18	19.4	15	0		Screaming fits, convulsions later

## 30 cc. undiluted evaporated milk:

244	6	15	20.0	13	6	40.8	Normal
249	6	18	18.2	21	5	40.1	1 rat died, possible convulsions
15	6	18	20.4	17	6	35.6	Normal
350§	5	22	25.8	10	5	39.4	Normal

## 40 cc. undiluted evaporated milk:

301	5	17	21.2	13	5	40.8	Normal
359	6	20	19.3	14	6	37.2	2 runts which showed slight signs of conv.
240	6	17	13.4	17	6	42.5	Normal

## Controls on Basal diet plus Auto. yeast only:

346¶	6	34			0		Screaming fits and convulsions
351	5	17			0		Screaming, running fits
30	5	19			0		Screaming fits, convulsions

\* Mother did not nurse litter well, so milk was given to her soon after birth. She then began to nurse the young.

† This litter was rather weak at time of transfer. Two of the young did not develop well and showed fits soon after transfer.

‡ Two of the young were smaller than the others and died after weaning.

§ This rat left some milk during part of experimental period.

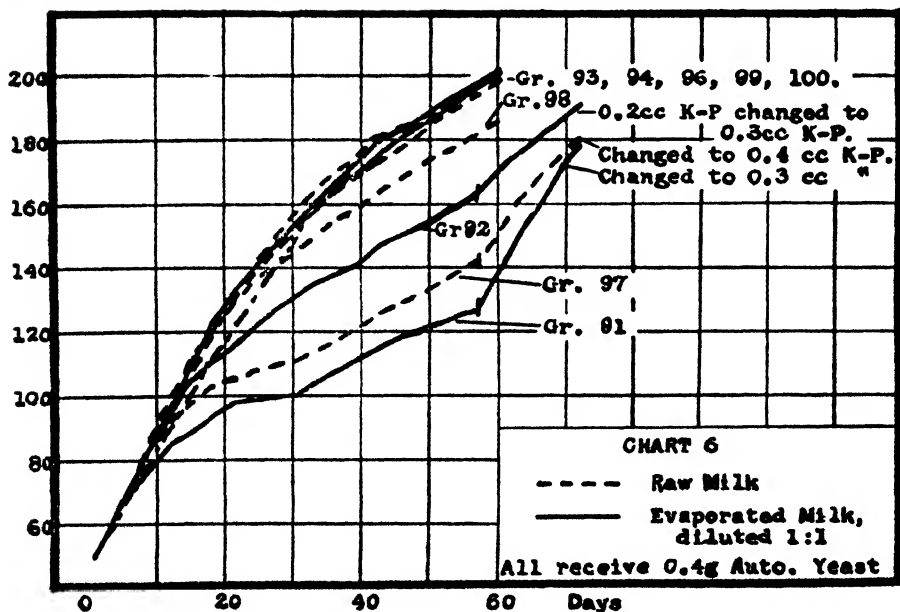
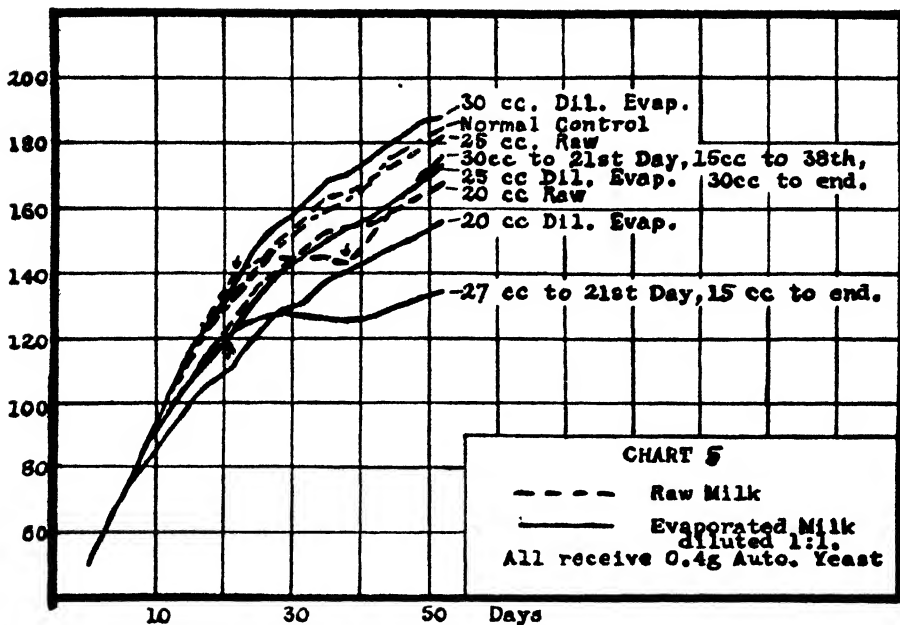
¶ Mother lost weight rapidly and developed severe polynuritis, but some of young while scrawny did not die, probably because they ate some of their dead mates.

within three to six days. When the weight of the litters became stationary, which occurred generally from the 14th to the 18th day, the material to be tested was fed in a separate container suspended from the top of the cage so that the young could not reach it. Litters were raised thus until they died or became large enough to reach the milk. The results of this experiment are given in Table II. The rats react somewhat variably, but a comparison of the records shows that the group on 50 cc. of raw milk raised most of the litters to an average weight of forty grams. The group receiving 30 cc. undiluted evaporated milk, equivalent to 60 cc. diluted, reacted similarly, but 25 cc. undiluted was too low. The ratio of the two milks with respect to their value for lactation is then of the same order as the ratios found in the growth experiments.

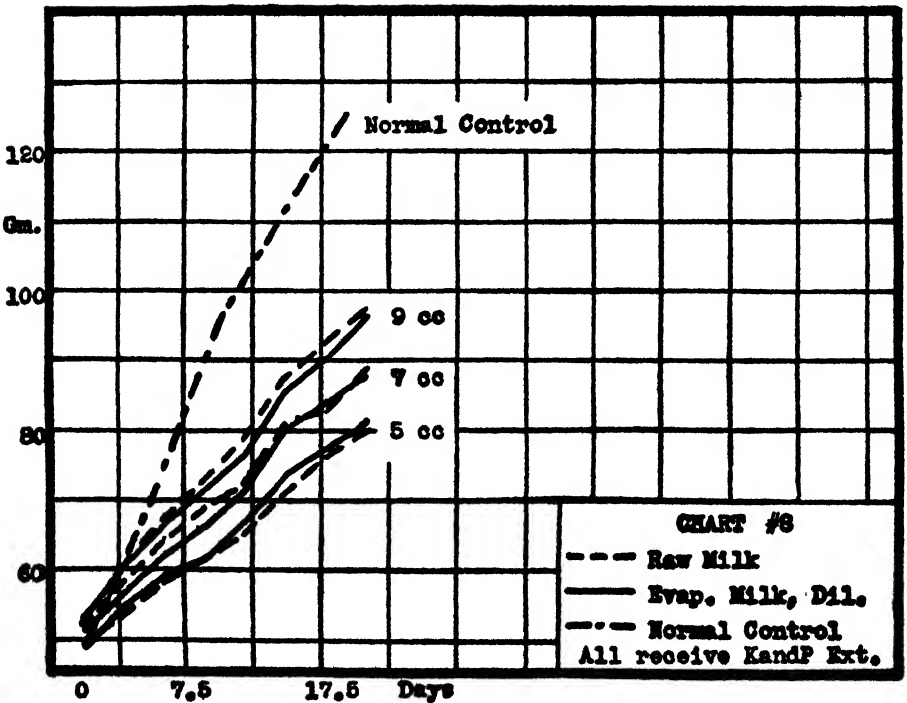
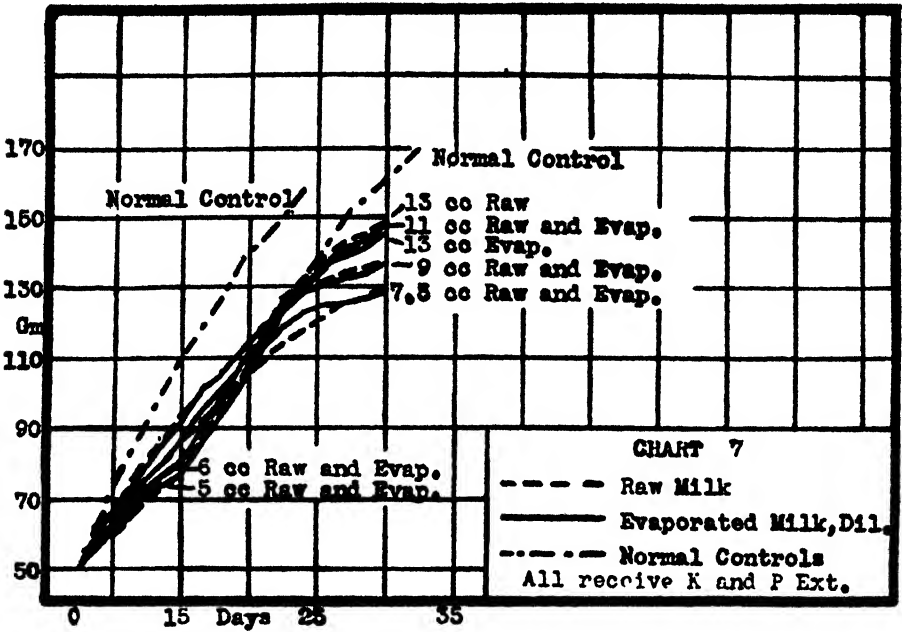
### *C. Measurement of the heat-stable fraction:*

Only one method has been used in this experiment, the method of adding various amounts of milk to a diet containing all constituents known to be essential for the rat, except the heat-stable fraction. The method was similar to that of Aykroyd and Roscoe (1) except that the growth periods were longer, parallel controls were always run, and rats were very carefully balanced. The method of adding a relatively pure extract has not been practicable here since it was found impossible to prepare concentrates from yeast which in small quantities would give normal growth to the adult stage. Concentrates prepared from the various fractions of the Kinnorsley and Peters residues varied widely in activity. In fact our results tended to confirm the evidence brought forward by Reader (25), Williams and Waterman (34), Hunt (16), and others, of a multiplicity of these factors. The original experiment consisted simply in the separate feeding of measured quantities of the raw and evaporated milks to carefully balanced groups of rats receiving an otherwise adequate ration made up of the basal diet plus a daily addition of a standardized antineuritic extract. In the second series the rats were even more carefully selected and the milk additions were all made up to the same volume to eliminate possible errors due to variation in fluid intake. The results are particularly significant in regard to the comparison of the two types of milk.

As was previously mentioned, the preliminary experiments indicated that the level of milk necessary to furnish adequate quantities of the heat-stable factor was lower than for the heat-labile factor. Consequently a series of 5, 6, 7.5 and 9 cc. of each milk (raw and 1:1 diluted evaporated) was begun. However, none of these amounts gave normal growth so after 17 days the groups on the two lower levels were increased to 11 and 13 cc. respectively. These quantities gave normal growth for some time but



then they too failed to keep up. Throughout this whole experiment however, as will be noticed in Chart 7, equal quantities of raw and diluted evaporated milk gave almost superimposable curves. It is also interesting to note that the 11 and 13 cc. levels did not separate significantly.



In the second series the quantities first selected for feeding lay in the subnormal range previously used at the beginning of the first experiment. This was done since it was at these levels that we could expect the widest spread per unit variation. Again as shown in Chart 8, the curves of the groups on equivalent quantities of raw and diluted evaporated milk coincided. Evidently the destruction by heat, if any, is less than can be distinguished by careful feeding experiments, since at no point do any pair of equivalent curves vary more than  $1/3$  the distance separating two intake levels at the time the quantities were changed. Since the difference between the two upper levels is only  $2/9$  the destruction must be less than ten per cent.

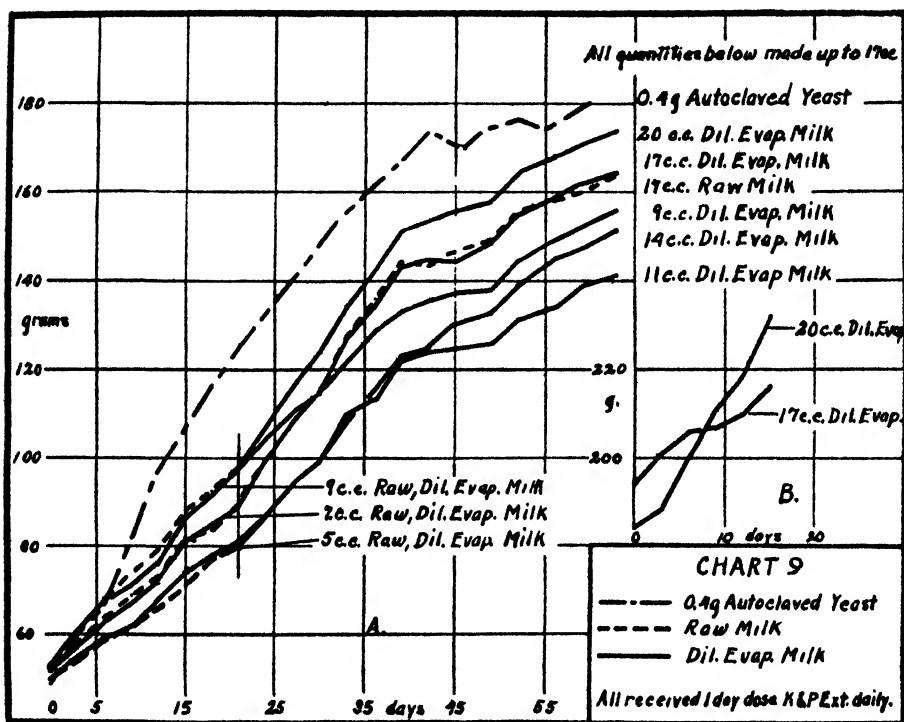
Again at the end of 21 days the quantities of milk were increased to all except one group of rats. Since it was clear by now however that there had been no significant destruction of the heat-stable fraction during evaporation, all groups except one were transferred to various levels of evaporated milk, thus giving a much wider range of levels. One group was continued on a higher level of raw milk, 17.0 cc., to be sure that no differences in the two milks indistinguishable at the lower levels might appear on the larger amounts. This curve practically duplicates its equivalent evaporated milk curve, even during a period of fluctuation in the growth of the colony, adding more conclusive evidence that there is no significant destruction of the heat-stable growth fraction during commercial evaporation.

The higher levels gave immediate increased growth as seen in Chart 9, the 17 and 20 cc. equivalents giving even slightly better growth than the control in the same region. The 17 cc. level gave as rapid growth as the 20 cc. level during the rapid period of growth but never reached the same level when growth slowed up. Since the two groups had been of different weights when transferred to the higher amounts, and consequently had grown at different rates during the first period, they should not have met during rapid growth but it was thought that all groups having adequate supplies would eventually reach the same level, the lower groups growing longer. On the other hand, conclusions could not be drawn from the period during which growth slowed up, since at this time we were unfortunately forced to use a new supply of the antineuritic solution which had not been well standardized, and at the same time to change technicians in charge of the rat feeding. That this was a serious disturbing influence is shown by the erratic behavior of the control group. It is impossible then from this series of curves to say whether 17 or 20 cc. of 1:1 diluted evaporated milk would be sufficient for normal growth and maintenance.

As further evidence on this particular question, two groups of rats previously used for standardization of antineuritic extracts were run on these



two higher levels. These do not have the same quantitative significance since the groups were smaller (3 rats) and were not so well balanced as far as litter mates of exactly the same weight were concerned. All were males. The results, shown in Curve 9B, would indicate that even 17 cc. of milk are not sufficient for optimum growth at these higher levels. The group which originally weighed less gained with sufficiently greater rapidity on the equivalent of 20 cc. of milk so that at the end it exceeded the weight of the group on 17 cc. of milk by more than its original negative difference.



However, it must be noted that this group gave better than our usual normal growth at this period.

The results of this series of experiments then indicate that raw and evaporated milk are equally good sources of the heat-stable factor and that the level of intake necessary for optimum growth lies between 17 and 20 cc. daily for the albino rats.

### DISCUSSION

The results in the growth experiments show that milk contains all the water-soluble vitamins necessary for the growth of the rat, but that rela-

tively large volumes must be taken to obtain optimum growth. Commercial evaporation has not seriously affected these substances. The small destruction in the case of the antineuritic fraction is probably in large part due to the process of sterilization in which temperatures above 100° C. are maintained for some time. Undoubtedly it is wise to include other sources of these vitamins in all infant dietaries.

Aside from this main point there are a number of very interesting things to be noticed.

When we began the series of lactation experiments, we considered the possibility of another factor being involved in lactation which might be so closely related to the antineuritic fraction that it might appear in the antineuritic extracts which had been used for curative effects. The symptoms of the young rats when first showing these effects are not the same as those seen in older rats on a polyneuritic diet (7, 15, 30). This might explain the discrepancy between the results of Daniels (7) and our growth experiments. However, our experiments indicated the same ratio between the two. Further than that, the rats which developed symptoms after longer growth often showed typical convulsive paralysis, while the younger rats of similar litters on the same diet showed the squealing running fits. Intermediate conditions occurred also. From this we were led to conclude that we were either dealing with the same substance or one which is destroyed at the same rate by heating.

Our observations of the relation of pellagra as a skin disease to the growth-promoting, heat-stable fraction were by no means clear. Like Sherman (27) we have found symmetrical loss of hair and brown scaling more often on our intermediate cases than on the controls totally lacking in the heat-stable fraction. In the latter we had rough, dull, but usually complete coats of hair, bloody irritated breakdown of the tissue around the nasal openings, chromogenic urine, and intestinal symptoms. Occasionally we have had severe skin lesions in these rats although they have been rare. On the other hand we have had case after case of loss of hair over large symmetrical areas with rats on 0.5–1.0 gm. of autoclaved yeast and growing rapidly. More careful correlative work between skin symptoms and growth must be done. However, no clear cut work can be well done until further purification of this fraction is carried out. The progress being made along this line should justify further study.

#### SUMMARY

Biological analyses by three different methods have shown that commercial evaporation of cows' milk destroys about one-sixth to one-fifth of the antineuritic heat-labile fraction. All cows' milk seems to be quite low

in this factor however, 25 cc. (3.49 gm. solids) being required per rat per day for optimum growth. Milks do not apparently vary widely in their antineuritic content as purchased on the market. There is no identifiable destruction of the heat-stable, growth-promoting fraction during commercial evaporation. Milk is capable of adequately supplying this fraction for optimum growth. The amount necessary, while lower than that required for the antineuritic factor, is also fairly large, that is 17 to 20 cc. (2.37 to 2.78 gm. solids). The question of the identity of this heat-stable factor with the antipellagra fraction is still unsettled.

The authors gratefully acknowledge the assistance of Miss Kathryn Knowlton in carrying out some of the quantitative feeding experiments. They also desire to express their appreciation for the coöperation given by the Evaporated Milk Association and the Carnation Milk Products Co.

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JULY, 1932

METABOLIC STUDIES IN A CASE OF  
OSTEITIS DEFORMANS\*

BY I. M. RABINOWITCH

*(From the Medical Service of Dr. C. P. Howard and the Department of Metabolism, The Montreal General Hospital, Montreal, Canada)*

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OSTEITIS deformans was first described by Sir James Paget of London in 1876. Although uncommon, it can hardly be regarded as a rare disease. In 1915, Da Costa *et al.* (1) reviewed the literature and found 213 cases, including 5 amongst their own records. Since then, the literature has greatly increased; there are to date 245 additional references. In 1925, Lewin (2) stated there were about 251 cases in the literature and on this basis Van Hazel and Andrews (3) concluded that the disease was rare. Since there were 213 cases prior to 1915, it would appear, according to Lewin's observation, that 38 cases only were reported between 1915 and 1925. As a matter of fact, the writer found 115, bringing the total number to 328 to the end of 1924. Since then 206 other cases have been reported, making a total of 534 to date. That the incidence of this disease is probably still greater is suggested from the rate of increase with the more general use of X-Rays. As is well known, X-Rays afford practically the only means of detecting this disease in its very early stages. With still more general use of this laboratory procedure, it appears reasonable that the incidence of this disease will probably be found to be still greater.

From the available literature, it is difficult to arrive at the true age and sex incidences, because these factors have received relatively little consideration in a large number of reports. A common practice appears to have been to report one case in detail and mention incidentally only the number of other cases met with. However, from the available data, the ages ranged from 6 to 89 years; the average was 56.8 years. There appeared to be more males than females; the ratio was about 1.5 to 1.

The pathology of this disease has frequently been dealt with in detail and, for purposes of brevity, will not be discussed here, except for relevant facts in the interpretation of our data. This communication is concerned

particularly with metabolic findings. Considering the number of reports, little consideration has been given to the metabolism of this disease. For example, though the intimate relationship between calcium, phosphorus and bone metabolism has been recognized for some time, only sixteen reports mention blood calcium studies and nine record blood phosphorus. Determinations of metabolic balances with respect to the inorganic elements are still fewer in number and the majority of them are relatively incomplete. For example, a few deal with calcium balance only and though others record calcium or phosphorus data, or both, no mention is made of sulfur. As Da Costa *et al.* point out, the changes in sulphur metabolism may be profound. The first and the most thorough investigation with regard to this phase of the disease was that of Da Costa, Funk, Bergeim and Hawk (1). More recently Cuthbertson reported balances of calcium, magnesium, phosphorus, sulphur and nitrogen (4). Both Da Costa *et al.* and Cuthbertson record essentially the same phenomena, namely, retention of calcium, magnesium and phosphorus and loss of sulfur. These findings appear to have been generally overlooked. Amongst the 245 new references, 28 only refer to the work of Da Costa *et al.* and such standard works as Sherman's Chemistry of Food and Nutrition, Lusk's Science of Nutrition and the most recent comprehensive work on "Quantitative Clinical Chemistry" (Peters and Van Slyke) make no mention of it. An opportunity was afforded for the investigation of the metabolism of a case met recently in the wards of The Montreal General Hospital and the purpose of this communication is to record our results. This patient was admitted into the Medical service of Dr. C. P. Howard, to whom I am very much indebted for his coöperation. The clinical details were, briefly, as follows:

#### Case History

A male, age 42 years, Hosp. No. 454/31, was admitted to this hospital on January 24th, 1931, complaining of pain in the chest, cough and "thickness of bones." The family history was irrelevant. The past history was also irrelevant, with the exception of "rheumatic fever" in 1913 and "frequent colds."

The present illness dates back to August, 1930, when weakness was first noticed. This was followed by loss of weight and pain in the chest. Since the beginning of the present illness he lost about twenty pounds.

The physical findings were negative, with the exception of some pyorrhoëa and dental caries, chronic tonsillitis and hypertrophy of the heart with a systolic murmur at the mitral area, the residual of rheumatic heart disease. The essential skeletal features were lordosis in the dorsal lumbar region, a hydrocephalic type of skull, moderate kyphosis and bowing of the legs. The laboratory findings were as follows:—

Blood: Red cells . . . . .	4,980,000
White cells . . . . .	6,500
Haemoglobin . . . . .	88 per cent

Differential:—polymorphonuclear leucocytes.....	63 per cent
lymphocytes.....	18 " "
eosinophiles.....	4 " "
monocytes.....	10 " "
myelocytes.....	2 " "
basket cells.....	3 " "
Wassermann.....	negative.

**Urine:** Clear: S.G. 1025: very faint trace of albumen; no sugar; no acetone bodies: occasional hyaline and granular cast and an average of 10 pus cells per high power field: pH 6.1

<b>Blood Chemistry:</b> Urea nitrogen.....	13 mgms. per 100 cc.
Creatinine.....	1.62 " " " "
Uric acid.....	3.47 " " " "
Calcium (serum).....	9.2 " " " "
Phosphorus (inorganic).....	3.84 " " " "
pH.....	7.41 " " " "
CO <sub>2</sub> combining power (plasma).....	58.5 vol. per cent.
Sugar.....	0.117 per cent.

**Blood Sugar Time Curve:** In the fasting state the urine was free of sugar and acetone bodies and the blood sugar was normal, namely, 0.117 per cent. One hundred grams of glucose were then given by mouth with the following results:

	Blood sugar	Urine sugar
30 minutes after ingestion....	0.200 per cent	0
60 " " " "....	0.169 " " "	0
120 " " " "....	0.125 " " "	0
150 " " " "....	0.100 " " "	0

**Electro-Cardiograph:** Left ventricular preponderance with evidence of myocardial damage.

**Basal Metabolic rate:**..... +6 per cent.

**X-rays:** The chest showed evidence of a low grade non-tuberculous infection. The heart appeared normal. The findings in the bones were as follows:

Both femora showed increased diameter and loss of differentiation between the medulla and cortex and striation characteristic of Paget's disease. There was no evidence of excess calcification.

The left humerus was normal. The right humerus showed increased diameter, chiefly confined to the sub-periosteal region. The medulla and cortex presented a striated appearance with scattered areas of rarefaction. A similar change was noted in the tuberosity.

Similar changes to that found in the right humerus were also noted in the eighth rib.

There was some rarefaction of the bone at the lower end of the right tibia, involving the medulla and cortex, but more marked in the cortex toward the anterior surface of the bone. There was no marked calcification.

### BALANCE OF INORGANIC ELEMENTS

The procedures (food, nursing, chemical methods, etc.) followed in our case conformed to our routine in such studies. As there is, perhaps, no other metabolism experiment in which more errors are likely to occur and nullify the value of data, an outline of this routine will be given briefly.

### NURSING

In spite of the greatest care possible on the part of nurses, even in the best of organized general hospital wards, there are a number of possible sources of error which may readily nullify results. In our experience, at least, a common source of error is carelessness on the part of orderlies in the transmission of food from the diet kitchen to the patient, or of specimens of urine and feces from the patient to the laboratory. Such errors may, unfortunately, never be detected and lead to publication of erroneous conclusions. As the data upon which they are based are faulty, they

confuse rather than enlighten when reference is made to them in future studies. In our routine, when a metabolic balance is to be obtained, the experimental subject is isolated from all other patients in the ward. Special day and night nurses are assigned who have no other duties but the care of the experimental subject. The patient is, thus, under *constant* observation. Orderlies are not allowed near the patient; the special nurses bring the food and return residues, if any, to the diet kitchen. These nurses are also responsible for the collection of urine and feces and their transportation to the laboratory. A urine refrigerator is kept at the patient's bedside.<sup>1</sup> There is, thus, no opportunity of specimens being mixed with those of other patients in the ward. Feces are collected in ordinary bed-pans and delivered *undisturbed* to the metabolism laboratory where the total weight of feces and pan is immediately obtained. A portion of feces is then taken for analysis, the pan is washed and the clean pan, after having been weighed, is returned to the ward. Carmine is used for partition of feces. To ensure collection of *exactly* 24-hour specimens of urine, the patient is given a drink of water every morning about one hour before the last specimen is to be collected, in order to promote a mild polyuria. When pans or urinals are necessary for collection of urine, they are rinsed with water when the urine is transferred to the jar in the refrigerator and the rinsing is added to the specimen. Creatinine determinations are used as a further check of the quantitative collections.

Volumes of urine and weights of feces are recorded immediately after the specimens are obtained. The specimens are then divided into two parts, as a protective measure against loss due to accidental breakage of container, etc.

#### MEASUREMENT OF FOOD INTAKE

Judging from the literature, it is not an uncommon practice, when determining metabolic balances, to make use of available food tables for calculations of food intake, though elaborate precautions may be taken otherwise with the remaining procedures. The possible sources of error inherent in such tables with food constituents other than the inorganic elements, were previously referred to (5). This criticism applies still more to the inorganic elements. The marked discrepancies possible between actual and assumed compositions are strikingly demonstrated when data of such tables are compared with those obtained by analyses of food materials actually used in a given experiment. As Sherman's tables (6) are most frequently employed, it may here be observed that the values recorded represent averages of whatever reasonable looking data the author was able to find at the time.<sup>2</sup> In some cases the averages were results of many determinations, while in others, the numbers were small. It may, therefore, be observed, that the statistical principle underlying the use of averages is that the arithmetical mean of a *large* series of observed values is the most probable value of the quantity measured;<sup>3</sup> the smaller the number of observations from which an average is calculated, the less is the significance which can be attached to that average. However, the author of these tables never intended that they should be used for such purposes. The purpose of compiling the data given was to afford approximate evaluations of intake when diets contain *many* articles of food and individuals are exposed to them over *long periods* of time. Under these conditions, there should be, as the author puts it, "a fair statistical probability of errors offsetting each other." In *short period* experiments, with use of a *small* number of articles of food, conclusions drawn from data suggesting loss or retention of the different elements are justified only when the chemical analyses include not only urine and feces, but, also, the different

<sup>1</sup> These refrigerators have been in use in this hospital during the last ten years and have proven highly satisfactory. They consist of wooden boxes 8×12×12 inches, lined with zinc or copper, and are partitioned in the middle. The jar containing a preservative rests in one section, while the other section contains ice. The cover is also lined with zinc or copper. They are not unsightly though, because of the number of diabetics, cardio-renal cases, etc., ten or more may be seen frequently in one ward.

<sup>2</sup> Personal communication.

<sup>3</sup> This is, of course, true only for a limited type, the symmetrical, of frequency distribution.

food materials ingested. In such experiments, repeated observations (unpublished data) have shown that differences of twenty-five per cent may very frequently be encountered between actual and assumed food values; and differences of as much as fifty per cent and more are not uncommon.

#### *Chemical Methods*

We do not use any of the micro-chemical methods in such studies, tempting as they may be. A disadvantage of macro-methods is that they require large amounts of material and are time consuming. However, in such experiments, the amounts of material available are quite large and as probable errors increase with the smallness of the amount of material used, micro-methods have no place in such procedures. An additional contra-indication is the fact, repeatedly noted, that these micro-methods, quantitative as they may be from a theoretical point of view, are not so practical, unless very exacting attention is paid to minute details. As the latter are time consuming, such methods offer no advantages over the standard macro procedures. In these studies all methods were gravimetric; calcium and magnesium were determined by McCrudden's procedures; calcium was isolated as the oxalate, the latter was then ignited and the calcium gravimetrically determined as calcium oxide; magnesium was isolated from the filtrates of the calcium determinations, after destruction of the organic matter, as magnesium ammonium phosphate; the latter was then ignited and the magnesium gravimetrically estimated as the pyrophosphate. For sodium and potassium estimations, the organic matter of the materials was oxidized according to the Neumann procedure. The sodium and potassium were then isolated as chlorides, converted into their respective platonic chlorides and their separation was based upon the practical insolubility of potassium-chloro-platinate in strong alcohol. Total sulfur in the urine was estimated by Benedict's method of oxidizing the organic matter with copper nitrate and potassium chlorate and gravimetrically determining the sulfur as barium sulfate. For sulfur of food and feces, the more recent procedures of Stockholm and Koch (7) were made use of. Phosphorus was determined by oxidizing the organic matter of the materials by the Neumann process. The phosphorus was then isolated as a phosphomolybdate and converted into magnesium ammonium phosphate. The latter was then ignited and the magnesium determined gravimetrically as the pyrophosphate. Chlorine was estimated by oxidizing the organic matter with sodium peroxide followed by precipitation of the halogen with silver nitrate and gravimetric determination of the silver chloride formed. All nitrogen determinations were made by the macro-Kjeldhal technic. The above procedures are all standard and, as details are available in a variety of works on quantitative chemistry, for purposes of brevity, no descriptions will be given here.

#### *Diets*

The diet in this case was liberal with respect to its carbohydrate, fat, protein and caloric content and composition of inorganic elements. As a matter of fact, according to available maintenance requirement data, excess quantities were largely used. The possible influence of such excesses will be dealt with later.

Important considerations in the interpretation of metabolic balances of the inorganic elements are the observations of Sherman and Gettler (8) and of Bogert and Kirkpatrick (9); the former showed that food, on burning, leaves an ash in which either acid or basic elements may dominate and the latter found, at least with respect to calcium, that acid ash causes a loss, whereas basic ash causes retention. The mode of elimination (urine and feces) appears also to be affected by the reaction of the ash; when the ash of the diet is basic, relatively less calcium is eliminated in the urine than when the ash is acid.

The above observations are referred to, in preference to those noted in animal experiments of a similar nature, for two reasons. Firstly, as they are the result of experiment in man, the data are more comparable. Secondly, the animal data are highly controversial. Thus, though there appears to be general agreement, that calcium-poor diets in the dog, are not conducive to equilibrium



of this element, a statistical investigation of many workers has failed to reveal any definite relationship (10). In spite of apparently carefully planned experiments, diametrically opposite results appear to have been obtained in experiments of a similar and simple type, such as measuring the effects of hydrochloric ingestion (11, 12).

The diet used in this investigation was constructed so as to yield an approximately neutral ash. In order to keep the number of necessary chemical analyses at a minimum, very few food materials were used. The following was the daily diet,—

	grams
Bread.....	420
Butter.....	85
Eggs.....	200
Milk.....	1680
Orange juice.....	200

Preliminary calculations of potential acid and basic ash of orange juice, milk and eggs were based upon the data given by Sherman and Gettler. For bread, calculations were based upon the amount of flour, salt, etc., used in the preparation of our hospital bread. The values for flour are given in the above mentioned tables.

In the following table, it will be observed, according to preliminary calculations, that the ash of the diet used was practically neutral; excess acid from the eggs and bread (corresponding to 53 cc. N/1 acid) was practically balanced by excess base from the milk and orange juice (corresponding to about 51 cc. N/1 alkali). Thus,—

FOOD  
Excess ash or base (cc. N/1 solution)

Type	Amount grams	Acid		Base	
		Per 100 grams	Total	Per 100 grams	Total
Eggs	200	11.1	22.2	—	—
Milk	1680	—	—	2.37	39.80
Bread	420	7.36	30.9	—	—
Oranges	200	—	—	5.61	11.22
Total			53.1		51.0

Since the use of food tables is not justified in such experiments for estimation of intake otherwise, it is obvious that they cannot be employed for estimation of ash. As with other analyses, it has been observed repeatedly that actual and assumed values rarely agree. It is also, obviously, not practical to determine reactions of ash before food is used in an experiment, as the analyses are time consuming and the food would, on standing, be unfit for consumption. Preliminary calculations such as the above are, however, of value in order to assist in constructing diets which *approximate* those required. From subsequent analyses of materials saved, the true reaction of the ash may be calculated and the results applied in the interpretation of data.

The method of calculating ash values was identical with that employed by Sherman and Gettler; the amounts of normal acid or alkali, corresponding to the amount of each element used, were calculated firstly. By adding together and balancing the results of all basic and acid forming elements, the daily ash was found to be slightly acid and corresponded to about 26 cc. of a N/1 solution.

TABLE I  
DAILY METABOLIC BALANCE, PERIOD 1 (FEB. 6 TO 11)

Day	Water Intake cc.	Water Output cc.	Urine creatinine nitrogen (grams)	Feces (grams)	Na gms. 3.39	K gms. 3.21	Ca gms. 2.45	Mg gms. 0.28	Cl gms. 5.82	P gms. 2.20	S gms. 1.39	N gms. 19.7	Daily intake ←
Feb. 6	3670	3430	0.560	None	2.97	3.45	0.46	0.103	5.63	1.61	1.26	17.8	Urine Feces Total
					—	—	—	—	—	—	—	—	
					2.97	3.45	0.46	0.103	5.63	1.61	1.26	17.8	
Feb. 7	2765	2275	0.586	201	3.21	2.87	0.11	0.081	5.21	0.87	1.42	16.4	Urine Feces Total
					0.29	0.31	1.62	0.066	0.26	0.45	0.21	2.6	
					3.50	3.18	1.73	0.147	5.47	1.32	1.63	19.0	
Feb. 8	2950	2460	0.496	225	2.21	3.11	0.33	0.073	6.31	1.11	1.16	17.0	Urine Feces Total
					0.13	0.11	2.29	0.052	0.17	1.38	0.18	2.9	
					2.34	3.22	2.62	0.125	6.48	2.49	1.34	19.9	
Feb. 9	3050	2610	0.543	150	3.56	2.96	0.76	0.064	5.13	0.62	1.43	16.7	Urine Feces Total
					0.17	0.28	1.35	0.193	0.25	0.34	0.49	2.9	
					3.73	3.24	2.11	0.257	5.38	0.96	1.92	19.6	
Feb. 10	3675	2490	0.560	None	2.82	2.67	0.32	0.118	6.89	0.86	1.62	17.7	Urine Feces Total
					—	—	—	—	—	—	—	—	
					2.82	2.67	0.32	0.118	6.89	0.86	1.62	17.7	
Feb. 11	2370	2850	0.505	201	3.96	3.31	0.26	0.042	5.02	0.68	1.61	17.9	Urine Feces Total
					0.08	0.39	1.29	0.062	0.37	0.49	0.27	2.6	
					4.04	3.70	1.55	0.104	5.39	1.17	1.88	20.6	

To avoid monotony, even during such short period experiments, special efforts are made by our Dietitian, Miss Ruth Parke, to vary the forms in which the few articles of food are served. For example, eggs are served as scrambled or in sandwich form, and eggs and milk are combined as egg-nogs, custards, etc.

#### RESULTS

The results, in detail, of the daily analyses of Period 1 of the experiment are shown in Table I and, briefly, summarized in Table II.

TABLE II  
RESUMÉ OF METABOLIC BALANCE. PERIOD 1.  
(All quantities in grams.)

	Na	K	Ca	Mg	Cl	P	S	N
Intake	20.34	19.26	14.70	1.680	34.92	13.20	8.34	118.2
Output: Urine	18.73	18.37	2.24	0.481	34.91	5.75	8.50	103.5
Feces	0.67	1.09	6.55	0.373	1.05	2.66	1.15	11.0
Total	19.40	19.46	8.79	0.854	35.96	8.41	9.65	114.5
Balance	-0.94	+0.20	- 5.91	- 0.826	+1 04	- 4.79	+ 1.31	+3.7
Percentage retention or loss	-4.5	+1.0	-40.2	-49.1	+ 2.9	-36.3	+15.7	+3.1
Percentage of total excretion in urine and feces.								
Urine	96.5	94.4	25.0	56.3	97.0	68.3	88.1	90.3
Feces	3.5	5.6	75.0	43.7	3.0	31.7	11.9	9.7

In the discussion of the data, consideration is given to (a) degrees of retention or loss of the different elements, (b) modes of their elimination (relative proportions in urine and feces), (c) possible relationships between amounts of material ingested and excreted and (d) the inter-relationships of the metabolism of the different elements.

#### *Sodium, Potassium and Chlorine*

From actual analyses of bone and from other available data, it would appear that the metabolism of sodium, potassium and chlorine differs from other elements to be considered presently; they not only play a small part in the formation of bone, but differ from the other elements also in that the body appears to be able readily to adjust itself to any tendencies towards their deficiencies, though they may not always be interchangeable and, in some of their functions, may actually be antagonistic; the "Law of the Minimum" appears to be able to assert itself readily. The operation of this law is strikingly shown in the experiments of Osborne and Mendel (13), those of Goodall and Joslin (14) and in Benedict's classical fasting

experiment (15). This probably explains the observation in our case of osteitis deformans that the patient was practically in equilibrium with respect to these elements. Their modes of elimination were also normal. Past experiences with such work suggest that such negative and positive balances as were found in this case are of limited significance. From repeated observations they appear to be transient and are apparently related to fluctuations of water content of the body—probably a compensatory mechanism in the regulation of osmotic pressure. They are not regarded as pathological.

*Calcium*

In the interpretation of the data with respect to calcium, appreciation of the intimate association between the metabolism of this element and bone formation is necessary. According to the table given by Sherman (6) on "The elementary composition of the body," calcium stands fifth in the list and constitutes about 1.5 per cent of the total body weight. It thus constitutes a larger proportion of the body weight than any of the other inorganic elements. The calcium content of body tissues is of interest here; though this element is required for a variety of physiological functions other than bone formation, (blood coagulation, etc.) there is a marked disproportion between its importance and the amount required for these functions; practically 99 per cent of all of the calcium is deposited in bone. About 85 per cent of all of the mineral matter of bone is represented by compounds of calcium (phosphate or carbonate) and the actual calcium content of normal human bone, expressed as calcium oxide, is about 28.8 per cent. McCrudden (16) gives the following percentage composition of normal human bone:

CaO. . . . .	28.85
MgO.....	0.14
P <sub>2</sub> O <sub>5</sub> .....	19.55
S.....	0.14

Differing from sodium and chlorine, the defensive mechanism against loss or an insufficient supply of calcium does not appear to be very perfect. This is shown in Benedict's fasting subject; on the last (31st) day of the experiment the amount of calcium excreted was still appreciable, namely, about one-half of that found on the first day; whereas, the excretions of sodium and chlorine were practically negligible. Thus:

Day	Urinary Excretion (gms.)		
	Na	Cl	Ca
1st.....	2.070	3.77	0.217
31st.....	0.053	0.13	0.138

Table II shows that there was a very marked retention of calcium in our case and the data agree essentially with those recorded by Da Costa *et al.* (1). In the two cases reported by the latter authors, the percentage retentions were 50.3 and 18.0 respectively.<sup>4</sup> As no two of these three patients were on the same diet, this must be considered in the interpretation. As our results are expressed in terms of the element alone, whereas those of the above mentioned authors are expressed in terms of calcium oxide, our data were re-calculated in terms of the oxide in order to make the findings comparable. The following table shows there was no relationship between intake and retention. Thus:

Subject	Calcium (CaO)	
	Intake per day (grams)	Retention (per cent)
W.....	2.585	18.0
B. (our case).....	3.430	40.2
McD.....	1.598	50.3

Normally, though restricted intake may lead to loss of body calcium (6), excess intake appears to have relatively little effect upon retention. In one of Sherman's experiments it was found that when the calcium content of the food was increased 250 per cent above the normal, 13 per cent only of the ingested calcium was retained (17). As the ingestions of calcium by the above mentioned subjects were not anywhere near those used in Sherman's experiment, some other explanation must be sought for the values found.

The relative proportions of calcium found in urine and feces are of interest. Normally, about 10 to 40 per cent of calcium is found in the urine, whereas the balance—by far the greater portion—is excreted by the intestinal tract (18). Food intake is an influencing factor; when the diet is rich in calcium, a large part of the latter is excreted by the intestines; whereas during fasting, or in conditions comparable to fasting (destruction of bone tissue, etc.) urinary calcium is increased. In one of Sherman's experiments when the calcium intake was very low, a large percentage of this element (42 per cent) was excreted in the urine. In Benedict's fasting man, during the period when no feces were passed, there was an appreciable excretion of urinary calcium. In a case of osteomalacia (19) about 68 per cent of the calcium excreted was found in the urine. A characteristic feature of this condition is destruction of bone with loss of calcium.

<sup>4</sup> Throughout the discussion of this paper these two cases will be referred to frequently as the method of their investigation was identical with that of our own case (nursing procedures, clinically, X-Rays and chemical methods). They will henceforth be designated as subjects W. and McD. respectively. Our case will be designated as subject B.

McCrudden (16) gives the following percentage composition of bone in this disease:

CaO. . . . .	15.44
MgO.....	0.57
P <sub>2</sub> O <sub>5</sub> .....	12.01
S. . . . .	0.55

Compared with the analyses given above for normal bone, the calcium content of bone in this disease was about one-half the normal.

The metabolic balance of a case of hyperparathyroidism—another condition which results in bone destruction—studied in this hospital (see Table V), showed that 73.4 per cent of the calcium was found in the urine.

In subjects W. and McD., the urinary calcium excretions, expressed as percentages of total excretions, were 4.6 and 1.0 respectively. In subject B. (our case) (Table II) about 25 per cent of calcium was excreted by the urine. Opposed to these findings are those made by Bruere for Gruner, Scrimger and Foster (20); in this case the urinary excretion was greater than the intake. However, in view of the fact that this case of osteitis deformans was complicated by multiple sarcoma formation and as the latter condition also causes loss of bone tissue, this case may be regarded as at least somewhat comparable to fasting during which body calcium is mobilized and relatively largely excreted by the kidneys.

How are these data to be interpreted? On the basis of X-Ray findings, Da Costa *et al.* (1) stressed calcification of arteries and of the pineal gland as a possible explanation of part of the calcium retention. In subject B., at least, the opinion is that the calcium retained was deposited chiefly in the bones; the X-Rays showed no calcification of arteries, and, with regard to the pineal gland, it may here be observed, as our Roentgenologist, Dr. W. L. Ritchie pointed out, that calcification is now regarded as a fairly common phenomenon; the incidence is about 30 per cent of all examinations made in this hospital to date. As a matter of fact, the condition is so common as to warrant little comment. It is of interest, however, to note, from actual chemical analysis, that though an increase of calcium in brain tissue was found to be uncommon, the calcium content was high in a case of Paget's disease (21).

The degree of retention of calcium in subject B. (our case) would suggest calcium starvation; the proportion of the total excretion found in the urine also corresponds to that found in starvation or destruction of bone, when the intake is normal. Since, as stated above, practically 99 per cent of calcium is found in bone, the most probable explanation is that, in osteitis deformans, there is simultaneously destruction of bone with loss of calcium

and an attempt at compensatory calcification. Available data are, however, conflicting. In subjects W. and McD., the X-Rays indicated calcification of the newly formed bone, but in subject B. (our case) there was no such evidence. An investigation of past records in this hospital, however, showed excess calcification in the majority of cases. Chemical analyses of bone also favor calcification; in all of the studies the calcium content was increased. Opposed to chemical analyses are histological data (22); these suggest little or no calcification. From an evaluation of the different methods of study (X-Ray, histological and chemical) it is, however, hardly necessary to state that chemical analyses afford the most reliable evidence. In other words, it may definitely be stated that excessive calcification is a characteristic of osteitis deformans.

The above incompatibilities become more apparent than real when due consideration is given to the bone pathology of the disease. As Adami and Nicholls (23) have pointed out, there are two opposing pathological processes, namely, (a) absorption of tissues and (b) osseous hyperplasia. With absorption of tissues there is bone destruction and replacement of bone by a fatty gelatinous or fibrous tissue, poor in cells; with osseous hyperplasia, there is proliferation and new bone formation in the periosteum and bone marrow which leads to increased mass and density. *Eventually* calcification occurs. The latter apparently represents the terminal (healing) process of this disease. As histological changes, X-Ray findings and the result of chemical analyses depend not only upon the degree but the extent and stage of the diseased process, it appears that all of the above data may readily be explained; in no two cases were the conditions comparable. Subject W. was apparently the mildest case and subject B. (our case) may be regarded as intermediate between subjects W. and McD.

It is obviously impossible to determine, with exactness, the severity of the disease. For practical purposes, however, it appears reasonable to assume that when the X-Rays show "rarefaction" only, the disease is more marked than when "striation" (osteoid formation) is noted; that the presence of subperiosteal deposits with increased diameter of bone indicates a less active lesion than the latter; and evidence of calcification suggests a healing, and therefore less severe, lesion. If these qualitative changes are correlated with the *number* of bones involved and the *extent* of the lesion in each bone, a classification with respect to severity appears possible. On this basis, the severity of the disease in the cases under discussion is, as stated above, as follows:

1.—W.

2.—B. (our case)

3.—McD.

Thus: In subject W., X-Rays not only showed calcification but involvement of two bones only, namely, the right tibia and upper end of the right femur; in subject B. (our case) there was no calcification, but more bones were involved; the lesions included both femora, the right humerus, the right tibia and the skull bones; whereas, in subject McD., though there was calcification, the disease was very widespread and included both clavicles, both scapulae, both humeri, both radii, both ulnae, the third metacarpal of the right hand, the index metacarpal of the left hand, many ribs, all of the lumbar vertebrae, all the pelvic bones, both femora, right patella, both tibiae, both fibulae, the astragalus and the os calcis on the right side. It is of interest to note that calcium retention and severity, according to the above method of classification, were parallel; the percentage retentions of calcium in the three cases were 18, 40.2 and 50.3 respectively.

The X-Ray finding of calcification in subject W., with a calcium retention of 18 per cent only and the absence of this change in subject B. with a 40.2 per cent retention of calcium may, at first, appear incompatible. An explanation suggested is that, though subject B. (our case) showed much more marked retention, the calcium was still, because of the more severe lesion, in some organic form and permeable to the X-Rays. Of interest with regard to the interpretation of this phenomenon are the observations of Taylor and Sheard as a result of their microscopic and X-Ray studies of calcification of tissues (24).

From the above it appears reasonable, though no observations have been recorded hitherto, to conclude that a metabolic balance obtained in the very early stage of the disease would probably show excess calcium excretion (i.e., loss) rather than retention; and a large proportion of this element would be eliminated in the urine. This may appear incompatible with the data in these three cases; the percentages of urinary excretion of calcium did not parallel severity; for subjects W., B. and McD. they were 4.6, 25 and 1.0 respectively. However, in no two of these subjects was the calcium content of the diet the same. Thus:

## CALCIUM OXIDE

Subject	Intake (gms.)	Output in urine (% of total)
W.	2.585	4.6
B. (our case)	3.430	25.0
McD.	1.598	1.0



This relationship between intake and excretion will again be referred to in the general discussion of the results.

### *Magnesium*

Interpretation of magnesium data is much more difficult than those of calcium, since very little is known of the physiological functions of this element. This probably explains the meagre consideration magnesium has been given clinically. That which is known, and relevant here, may be briefly summarized.

That magnesium is intimately concerned with the metabolism of bone is suggested from the fact that about two-thirds of the total amount in the body is found in this tissue (25). It exists in bone as the solid phosphate and carbonate. According to the above analysis, given by McCruden, it forms a very small fragment of the solid inorganic matter of bone. Differing from calcium, magnesium appears to be very widely distributed in nature and the body appears able to adapt itself readily, as in the case of potassium, sodium and chlorine, to tendency towards deficiencies. With regard to its elimination, again differing from calcium, the kidneys and intestines play an approximately equal part, under normal conditions (18). There, however, appears to be an intimate relationship between calcium and magnesium metabolism, at least with respect to bone. Bogert and McKittrick (26) have shown that excessive intake of calcium may lead to excessive excretion of magnesium and vice versa. In spite of this antagonism, deposition of magnesium in bone is presumably determined by the same factors which account for deposition of calcium (27). This would suggest itself from the closely allied chemical nature of these two elements, according to their position in the Mendeljeff table. This probably also explains the observation that one element can replace the other. For example, in osteomalacia (19), loss of calcium was accompanied by retention of magnesium and the same phenomenon was observed in our case of hyperparathyroidism (Table V). The metabolic balance in the latter case showed a 50 per cent retention of magnesium. There is, also, indirect evidence (28, 29) that, though calcium and magnesium may not ordinarily subserve the same function with the organism, they can, apparently, to a certain extent at least, replace one another. It is of interest, here, to note that another element in the same column of the table of elements as magnesium has been found able to substitute for calcium, namely, strontium (30). This phenomenon will again be referred to in the interpretation of the data.

The data in subject B. are interpreted on a basis of the above observations. On a daily intake of 2.772 grams of magnesium oxide there was marked retention, namely 49.1 per cent. Again, if the three subjects are arranged in order of severity, there appears to be some relationship between the latter and degree of retention. Thus:

Subject	Daily intake (gms.)	Percentage retention
W.	4.229	35.1
B. (our case)	2.772	49.1
McD.	2.768	58.7

As the greater part of the magnesium is in bone, as the factors which govern deposition of calcium also govern that of magnesium, and as magnesium may substitute for calcium when the latter is in great demand, interpretation of the above findings does not appear difficult.

There appears to be no abnormality with respect to the mode of elimination; slightly over one-half of the total amount of magnesium eliminated was excreted by the kidneys.

### *Phosphorus*

The intimate relationship between the metabolism of phosphorus and bone formation is obvious. Phosphorus is the limiting factor in bone formation. About 20 per cent of bone is composed of phosphorus, calculated as  $P_2O_5$ . It and calcium are the essential constituents of the rigid supporting structure of all osseous tissue. It has been calculated that the body contains about 700 grams of phosphorus and 600 of the latter is found in bone. Though phosphorus may exist in many forms (nucleo-proteins, phosphatides, in combination with starch, etc.), it appears, from available data, that it is permissible to compute the total phosphorus intake in a metabolic balance experiment without regard to separate computation with respect to the different forms and different sources of this element. From the point of view of bone pathology, it is of interest to note that, as in the case of calcium, compared with chlorine, sodium and potassium, the body is poorly fitted to cope with tendencies toward deficiencies; liberal amounts of phosphorus are always required to maintain equilibrium.

As with calcium and magnesium, there was a marked retention of phosphorus in subject B. On an intake of 5.060 grams, calculated as  $P_2O_5$ , there was a retention of 36.3 per cent. Again, there appears to be some relationship between the severity of the disease and degree of retention. Thus:

Subject	Daily intake (gms.)	Percentage retention
W.	4.300	28.6
B. (our case)	5.060	36.3
McD.	3.972	50.3

As the excretion of phosphorus is greatly influenced by the rate of metabolism and intake (6), these factors are excluded in subject B. at least; the intake was not only sufficient under ordinary conditions, but slightly in excess; the basal metabolic rate was also normal (+6 per cent).

With regard to the mode of elimination, here, there was nothing abnormal to note; about 65 per cent was excreted by the kidneys and the remainder by the intestines.

### *Sulfur*

The sulfur metabolism is of particular interest in that, differing from the other elements, the metabolic balance showed a definite loss (Table II) and, as with the other elements, there appeared to be some relationship between severity of the disease and excretion. Thus:

Subject	Daily intake (gms.)	Percentage
W.	0.769	2.3 retention
B. (our case)	1.39	15.7 loss
McD.	0.612	43.8 loss

The sulfur metabolism data are as difficult to interpret as those of magnesium, also because of our limited knowledge of the physiology of this element. Analysis of bone (McCrudden) shows that it contains very little of this element. It is very difficult to attribute loss of sulfur to destruction of bone alone, since, in osteomalacia, sulfur was found to be retained rather than lost (16,19) and in our case of hyperparathyroidism (Table V) there was also a retention of sulfur.

Difficult to reconcile are the nitrogen and sulfur data. Under ordinary conditions, the amount of urinary sulfur excreted tends to parallel protein catabolism. This is the basis of the use of the so-called N:S ratio. This ratio is, however, not so constant as was originally supposed; it has been found to vary between 5:1 to 13:1 or more, and is affected by a variety of conditions. Thus, in fasting, the value is lower in the early stages than in the late period, suggesting, as Peters and Van Slyke point out (27), that nitrogen in excess of sulfur is preferentially being retained in the body. This attempt, during fasting or conditions comparable to fasting with respect to loss of body tissue, to retain nitrogen is also sug-

gested from the following data in the three cases of osteitis deformans. Thus:

Subject	PERCENTAGE RETENTION OR LOSS	
	Sulfur	Nitrogen
W.	2.3 retention	1.0 retention
B.	15.7 loss	3.1 loss
McD.	43.8 loss	1.06 loss

#### INTERRELATIONSHIP OF ELEMENTS

A number of workers have calculated ratios of one element to another in an attempt to interpret the data obtained in metabolic balance experiments in osteitis deformans. For example, the ratio of calcium to magnesium has been found similar to that of growing bone (1) and, on the basis of observed ratios of nitrogen to sulfur, attempts have been made to differentiate the character of the organic matrix laid down in diseased bone from that of normal bone (19). Our ratios, when calculated, do not agree with the different reports and an explanation of the differences is suggested in the following observations:

The type of metabolic balance experiment indicated in a given case depends upon the information sought. For example, as Sherman points out (6), "maintenance requirement" experiments differ from those when studying "requirements during growth" or disease associated with abnormal demands of the different elements. With respect to the element under consideration, in order to determine maintenance requirements, the intake and output are measured on diets of normal character, but low in that element, until one finds the minimum amount which will just permit the maintenance of equilibrium. On the other hand, in studying the phenomenon of growth, the plan is to find the intake which will support an optimum rate of storage of the element in question in the growing body. As far as we have been able to ascertain, experiments corresponding to those required to interpret ratios of one element to another have not been performed in cases of osteitis deformans; all have been of the same type as our own. In view of this fact and our lack of information with respect to the selective properties of bone in this disease when exposed to diets containing different mixtures of the different elements, it is obvious that limited significance must be attached to the ratios recorded.

An additional difficulty with regard to the interpretation of such ratios as calcium to phosphorus and magnesium to phosphorus is the fact that bone is not absolutely constant in its inorganic composition; both ratios of one element to another and one compound to another have been found

TABLE III  
DAILY METABOLIC BALANCE, PERIOD 2 (MAR. 9 TO 14)

Day	Water Intake cc.	Water Output cc.	Urine creatinine nitrogen (grams)	Feces (grams)	Na gms. 3.61	K gms. 3.28	Ca gms. 2.25	Mg gms. 0.29	Cl gms. 5.92	P gms. 2.10	S gms. 1.38	N gms. 21.7	Daily intake
Mar. 9	2470	2160	0.513	None	3.33 — 3.33	2.65 — 2.65	0.21 — 0.21	0.087 — 0.087	5.36 — 5.36	0.92 — 0.92	1.46 — 1.46	20.6 — 20.6	Urine Feces Total
Mar. 10	2960	2680	0.486	130	3.11 0.26 3.37	3.16 0.22 3.38	0.63 1.03 1.66	0.062 0.074 0.136	5.29 0.37 5.66	0.75 0.46 1.21	1.68 0.22 1.90	19.9 1.0 20.9	Urine Feces Total
Mar. 11	3085	2650	0.453	103	4.21 0.26 4.47	3.46 0.11 3.57	0.29 0.94 1.23	0.053 0.096 0.149	6.13 0.21 6.34	1.34 0.63 1.97	1.21 0.13 1.34	20.0 2.1 22.1	Urine Feces Total
Mar. 12	2560	2460	0.534	100	3.86 0.11 3.97	3.33 0.06 3.39	0.32 1.26 1.58	0.046 0.096 0.142	5.87 0.16 6.03	2.24 1.18 3.42	1.63 0.23 1.86	19.3 2.2 21.5	Urine Feces Total
Mar. 13	2500	2650	0.503	35	3.65 0.07 3.72	2.68 0.11 2.79	0.16 1.97 2.13	0.098 0.141 0.239	5.93 0.12 6.05	0.63 0.55 1.18	1.67 0.18 1.85	19.8 1.6 21.4	Urine Feces Total
Mar. 14	2850	2480	0.469	110	3.10 0.12 3.22	3.21 0.47 3.68	0.09 0.87 0.96	0.092 0.106 0.198	5.64 0.38 6.02	0.92 0.83 1.75	1.29 0.16 1.45	20.3 1.5 21.8	Urine Feces Total

to vary widely. The picture is complicated still further by recent work which suggests that calcium and phosphorus may be deposited in bone, not as  $(Ca)_3(PO_4)_2$  but as  $Ca H PO_4$ . It has been claimed that solubility products in blood and the composition of bone agree better with this hypothesis than with the generally accepted idea. The microscopic and X-Ray investigation of calcification of tissue by Taylor and Sheard are, however, of particular interest here; X-Rays and refractive methods have revealed no secondary phosphate in bone.

A metabolic balance was, again, obtained about one month later; the phenomena observed were essentially the same. The details of this experiment are shown in Table III and, briefly, summarized in Table IV. During the interval between the two experiments, the patient received an intensive course of quartz light treatment. The latter, obviously, was without effect, at least with respect to the inorganic elements. This is of interest with regard to vitamin deficiency as a possible cause of this disease. This possibility will be referred to under the discussion of "etiology."

TABLE IV  
RESUMÉ OF METABOLIC BALANCE. PERIOD 2.  
(All quantities in grams.)

	Na	K	Ca	Mg	Cl	P	S	N
Intake	21.66	19.68	13.50	1.740	35.52	12.60	8.28	130.3
Output: Urine	21.26	18.49	1.70	0.438	34.22	6.80	8.94	119.9
Feces	0.82	0.97	6.07	0.513	1.24	3.65	0.92	8.4
Total	22.08	19.46	7.77	0.951	35.46	10.45	9.86	128.3
Balance	+0.42	-0.22	-5.73	-0.789	-0.06	-2.15	+1.58	-2.0
Percentage retention or loss	1.8	1.1	42.4	45.3	0.2	17.0	19.0	1.3

Percentage of total excretion in urine and feces.

	96.2	94.8	21.8	46.0	96.2	65.0	90.6	93.4
Urine	96.2	94.8	21.8	46.0	96.2	65.0	90.6	93.4
Feces	3.8	5.2	78.2	54.0	3.8	35.0	9.4	6.6

### *Etiology*

In their discussion on etiology, Da Costa *et al.* (1) suggest a number of possibilities; and these include disturbances of the ductless glands. Since their report in 1915, however, material advance has been made in the study of internal secretions (parathyroid, thyroid, pancreas; adrenals, pituitary, etc.) and, though our knowledge is by no means complete, it appears possible to exclude a number of conditions. Material advancement

has also been made with respect to vitamins and the conditions which govern acid-basic equilibrium, and, as is well known, both of the latter influence the metabolism of the inorganic elements.

There appears to be little doubt that inorganic metabolism can be influenced by the thyroid gland; thyroid feeding and overactivity (hyperthyroidism) cause increased excretion of calcium and phosphorus; whereas hypothyroidism has a diametrically opposite effect (31). However, the sulfur metabolism in the above conditions appears to differ fundamentally from that in osteitis deformans; it, apparently, bears little or no relationship to the metabolism of the other inorganic elements; sulfur excretion appears to be dependent upon protein metabolism alone; excretions of sulfur and nitrogen are parallel and the N:S ratio is constant. This probably explains the finding of a constant ratio of N:S in spite of excess calcium excretion after nitrogen equilibrium was established (32). Hyperthyroidism and hypothyroidism are also excluded on the basis of the clinical condition and basal metabolic rate data. Boothby and Sandiford (33) found the basal metabolic rate normal in five of six cases of osteitis deformans and in our case (subject B) it was also normal, namely +6 per cent. The blood sugar time curve also tends to exclude hyperthyroidism.

TABLE V  
RESUMÉ OF METABOLIC BALANCE IN A CASE OF HYPERPARATHYROIDISM  
(Period of observation 10 days.)  
(All quantities in grams.)

	Na	K	Ca	Mg	Cl	P	S	N
Intake	34.81	35.63	21.67	2.54	58.50	21.40	12.40	197.0
Output: Urine	30.30	30.67	20.04	0.77	57.34	18.48	9.15	174.0
Feces	2.18	2.74	7.23	0.44	2.71	9.52	1.52	13.5
Total	32.48	33.41	27.27	1.21	60.05	28.00	10.67	187.5
Balance	-2.33	-2.22	+5.60	-1.33	+1.55	+6.60	-1.73	-9.5

Percentage of total excretion in urine and feces.

	93.2	91.8	73.4	63.6	95.4	66.0	85.7	92.8
Urine	93.2	91.8	73.4	63.6	95.4	66.0	85.7	92.8
Feces	6.8	8.2	26.6	36.4	4.6	34.0	14.3	7.2

Though, as stated above, it is quite possible, in the early stages of osteitis deformans, that the disease is associated with loss of calcium, and thus agrees with hyperparathyroidism, other features, particularly the metabolic balance shown in Table V, appear to exclude excessive activity of

the parathyroids as a cause; from the stage of the disease and number of bones involved, the lesion in our case of hyperparathyroidism differs little with respect to its severity (according to the above mentioned classification of severity) from the case of osteitis deformans. The results of the metabolic balances differed, however, very greatly; in the former case there was loss of calcium and retention of sulfur; whereas in the latter there was retention of calcium and loss of sulfur. Incidentally, the serum calcium was markedly increased in the former case, namely, 14.2 mgms. per 100 cc., whereas in the case of osteitis deformans it was normal (9.2 mgms. per 100 cc.). Koechig (34) reports high calcium values obtained in two cases, namely, 12.1 and 11.8 mgms. per 100 cc. However, Lyman's method (35) and citrated blood were used. It may here be observed that plasma tends to yield higher values than serum.

With regard to the known internal secretion of the pancreas (insulin) our records show two cases only of Paget's disease, amongst over 3000 patients in the diabetic clinic of this hospital. On a statistical basis, therefore, this is excluded as a possible cause.

The clinical picture (complaints, pulse, blood pressure, skin, etc.) and laboratory data (B.M.R., sugar tolerance, etc.) exclude, at least obvious hypo- and hyper-adrenalinism.

The practically neutral diet, pH of blood,  $\text{CO}_2$  combining power of the plasma and urine acidity, exclude disturbance of acid-basic equilibrium as a cause.

Vitamins were considered as a possible etiological factor. That vitamins may affect the metabolism of bone is too well known to require further comment. The intimate relationship between vitamin D and calcium metabolism is an example. However, at least in our case, the environment of the patient and his dietary habits appear to exclude osteitis deformans from the group of vitamin deficiency diseases.

Abnormal enzyme (phosphatase) activity was considered as a possible factor. The observations of Kay are of interest here (36). The enzyme phosphatase has apparently the property of hydrolysing soluble calcium salts of phosphoric esters to insoluble calcium phosphate. It is present in bone in localities in which deposits of calcium phosphate are proceeding most rapidly. Excessive plasma phosphatase appears to be confined to bone disease and the highest values were found in osteitis deformans. Phosphatase has no diagnostic significance; it does not differentiate the forms of bone lesions. The conclusion, however, to be drawn from available data to date is that the high values found in bone disease are probably the result rather than the cause of the bone lesions.



The conclusion to be drawn from the above observations is that the etiology of osteitis deformans is, as yet, unknown. The probability, however, is that it belongs to the "metabolic" diseases rather than due to infection. Interpretation of the inorganic balance is difficult; calcium, magnesium and phosphorus data appear to depend upon the stage, severity and extent of the disease and similar findings may be met with in other bone lesions. The sulfur metabolism, however, appears to be a differentiating factor, being confined exclusively to osteitis deformans.

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## THE ADDITION OF RAW BEEF OR MEAT SCRAP TO A WHEAT-MILK DIET\*

BY WALTER C. RUSSELL

*(From the Department of Agricultural Biochemistry, New Jersey  
Agricultural Experiment Station, New Brunswick)*

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**I**N MAY, 1926, a group of albino rats was obtained from Wistar Institute, Philadelphia, and placed upon Sherman Diet B (1) which consists of 2/3 ground whole wheat, 1/3 dried whole milk, with the addition of sodium chloride to the extent of 2 per cent of the weight of the wheat. Although they continued to grow and young were born, the number per litter was small and the weight of those raised to the weaning age, 28 days, was less than that desirable for nutritional studies. Of 11 pregnant females, out of 12 mated, 3 ate their litters and the remaining 8 produced 53 young. Of these, 45 were weaned at 4 weeks of age and placed upon Diet B, but 27 of them died during the 2 weeks immediately after weaning. The condition of those that died was characterized by a sluggishness, failure to eat and a wetting about the pubic area.

The fact that the dead or dying young were eaten by their litter mates suggested the feeding of raw meat and workers in other laboratories informed us that raw meat sometimes brought about improvement in the condition of a breeding colony. Sherman (2) in 1925 mentioned the feeding of raw beef to mother rats as a supplement to Diet B and later MacLeod (3) reported improvement in reproduction when fresh meat or dried meat was added to certain diets. Hence the practice of feeding 5 gm. of raw beef steak per adult rat in the breeding colony, daily, except Sunday, was begun. Two weeks after the beginning of the meat feeding the same 12 females were again mated. Ten litters were produced totalling 89 living young and 2 dead. In some instances the litters were reduced to 7 young but none died of natural causes before the weaning ages, 21 or 28 days. After weaning they were used successfully for nutritional studies and for breeding.

The improvement in the condition of the breeding stock and of the young produced in the preliminary trials with raw beef, led to the regular practice of feeding it, as a supplement to the Sherman Diet B, to the breeding colony and to young animals selected as breeding stock. Individual

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portions were not given to the nursing young but they obtained small amounts from the portion given to the mother. Since by far the greater part of the meat was consumed by the mother it is assumed that the improved condition of the young was due, in part at least, to improved lactation. A summary of the results obtained when the raw beef supplement was fed is given in Table II, Diet BM.

This paper reports our experience with the unsupplemented Sherman Diet B, with this diet supplemented with raw beef, Diet BM, and with a meat scrap incorporated in the diet, Diet BMS.

### COLONY MANAGEMENT

It is our practice to mate young breeding stock for the first time at 120 days of age and to use them for breeding until 4 litters have been obtained. This is usually accomplished in a period of 10 months, at least a 2-week's rest period being allowed between weaning and the next mating. The mating ratio has been 1 male to 2 females. Only males weighing 290 gm. to 300 gm. or more, and females weighing 190 gm. to 200 gm. or more at 120 days are used for the breeding colony.

During the first week of life, usually within the first 3 days, the number of young in a litter is reduced to 7. If a female in good condition is available with a litter of the same age as that to be reduced, but less than 7 in number, young are given to her to nurse. The reduction to 7 in a litter was not practiced when the colony was first started but it has been the custom during the greater part of the time covered by this report.

If a female fails to become pregnant after being with a male for 4 weeks, she is placed with another male for the same length of time. If after a third mating she does not become pregnant she is considered sterile and is discarded. Each placing with a male, whether it resulted in a pregnancy or not, is counted as a mating.

### EXPERIMENT 1

In order to find out whether some other supplement could be used in place of raw beef, preliminary feeding trials were made with small groups of animals in which meat scrap<sup>1</sup> and dried yeast were compared with raw beef as supplements to Sherman Diet B. A group receiving unsupplemented

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<sup>1</sup> According to the Purdue Univ. Agric. Expt. Sta. Circ., No. 147 (1927), "Meat scrap and meat meal are ground residues from animal tissues, exclusive of hoof and horn, and contain less than 10 per cent phosphoric acid ( $P_2O_5$ ).'' Swift's meat scrap, 55 per cent protein, was used in the present study.

Diet B was also used. The animals employed were born and reared to weaning at 28 days on Sherman Diet B supplemented with raw beef. Immediately upon weaning they were placed upon the experimental régime. All groups were started at the same season of the year, September, 1927, and mated at 4 months of age.

Each experimental group received Sherman Diet B *ad libitum*. To Group 1 was given a supplement of 5 gm. of fresh raw, lean beef per rat per day. Each individual of Group 2 received at first a daily supplement of 1 gm. and later of 2 gm. of meat scrap until 4 months of age when 10 per cent was incorporated in the diet. Dried yeast, 300 mg. per day, was given to each member of Group 3. A supplement was not given to Group 4.

An attempt was made during a 5-months' period to rear 2 litters to 21 days of age in the case of each female. This was accomplished in the meat and meat scrap groups by the first and second matings, but in the other two groups a larger number of matings was necessary, as indicated in Table I. The average number born per litter in the control group was the smallest of the 4 groups. Furthermore, the average weight of young of the yeast and control groups, at 21 days of age, was definitely less than in the instance of the meat and meat scrap groups, and too low for experimental purposes. At 4 months of age the animals raised for breeding on the meat and meat scrap diets were considerably heavier than those of the other two groups.

Although only small numbers of individuals were employed, the results are quite conclusive that growth of breeding stock to the mating age, reproduction and growth of young are markedly better when raw beef or meat scrap is used with Diet B. The meat scrap is equivalent to raw beef in these respects and both are superior to dry yeast. Sherman and Campbell (1) note that Diet B "is probably capable of further improvement."

## EXPERIMENT 2

On the basis of the results of Experiment 1, 10 per cent of meat scrap was incorporated in Sherman Diet B, at the proportionate expense of the wheat and milk, for part of the breeding colony. The confirmation of the preliminary trials led to the discontinuance of the feeding of fresh meat and the exclusive use of Diet BMS. In practice the diet used in the colony, Diet BMS, consists of 60 per cent ground whole wheat, 30 per cent dried whole milk<sup>2</sup> and 10 per cent meat scrap (Swift's) with the addition of sodium chloride to the extent of 2 per cent of the weight of the wheat.

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<sup>2</sup> Klim.

TABLE I  
RESULTS OF PRELIMINARY SUPPLEMENTATION TRIALS

No. of animals	Sex	Av. weight at 28 days†	Av. weight at 4 mos.	No. of matings	No. of pregnancies	No. of litters	No. of young born	Av. no. born per litter	No. alive at 21 days*	Av. no. per litter at 21 days*	Av. weight at 21 days
gm. gm. Group 1, Sherman B+raw beef (Diet BM)											
4	M	68	361	12	12	12	122	10.1	77	6.4	43
6	F	64	225								
gm. gm. Group 2, Sherman B+meat scrap (Diet BMS)											
4	M	63	324	10	10	10	108	10.8	69	6.9	44
5	F	65	211								
gm. gm. Group 3, Sherman B+yeast (Diet BY)											
5	M	65	271	9	6	6	68	11.3	42	7.0	32
4	F	63	188								
gm. gm. Group 4, Sherman B (Diet B)											
5	M	63	278	15	11	10	75	7.5	35	5.9	27
5	F	59	188								

† On Diet BM until weaning.

\* Litters were reduced to 7 young during the first week of life.

Although the use of the above modification of the Sherman Diet B has been successful, as demonstrated in Tables II and IV, another attempt was made, beginning in January, 1930, to use the original Sherman Diet B. This trial was made after the breeding colony had been on Diet BMS for 2 years and the young used were born and reared to 28 days on this diet. At weaning, 28 days, 38 female litter mates from 15 litters were divided into groups of 19 each, one of which received Sherman Diet B and the other Diet BMS. The males were selected from the same group of litters so that litter mates appeared in each of the experimental groups. Mating was begun at 120 days or shortly thereafter and was conducted so that each female had an opportunity to have two litters.

TABLE II  
SUMMARY OF REPRODUCTION AND GROWTH RECORDS ON DIETS BM, BMS AND B\*

	Diet BM	Diet BMS	Diet B
No. of females at 28 days . . . . .	47	159	26
Av. weight at 28 days . . . . .	55.6 gm.	62 gm.	57 gm.†
No. of females at 120 days . . . . .	45	156	26
Av. weight at 120 days . . . . .	196 gm.	198 gm.	183 gm.
No. of litters observed . . . . .	108	405	41
Total no. of young observed . . . . .	903	3600	261
Total no. of young observed alive . . . . .	869	3361	220
Per cent of total observed alive . . . . .	96.2	93.5	84.3
Av. no. of young alive per litter at birth . . . . .	8.0	8.3	5.4
Percentage of young born alive which died during first 7 days of life from natural causes . . . . .	5.7	6.2	61.9
Nursing at 7 days of age:			
No.‡ . . . . .	702	2476	70
Av. weight . . . . .	14.0 gm.	14.0 gm.	9.8 gm.
Nursing at 21 days of age:			
No. . . . .	656	2437	66
Av. weight . . . . .	40.5	40.6	25.6

\* Includes animals reported in Tables I and III.

† Born and reared to 28 days of age on Diet BM or BMS.

‡ Litters reduced to 7 young during 1st week.

The results are essentially those observed in previous trials and are summarized in Table III. The colony routine of discarding males less than 290 gm. to 300 gm. in weight, and of females of less than 190 gm. to 200 gm. in weight at 120 days, was practiced with regard to the animals on Diet BMS but not in the case of Sherman Diet B because only a few reached this standard. Litters were observed within 18 hours after birth.

TABLE III  
EXPERIMENT 2  
COMPARISON OF REPRODUCTION AND GROWTH RECORDS, DIETS B AND BMS

	Diet B	Diet BMS
At 28 days of age:		
No. of females . . . . .	19	19
Av. weight . . . . .	56.3 gm.†	58.5 gm.
At 120 days of age:		
No. of females used for mating . . . . .	19	15*
Av. weight . . . . .	180 gm.	208 gm.
No. of litters observed . . . . .	25	27
Number born:		
Observed living . . . . .	139	262
Observed dead . . . . .	36	2
Average number observed alive per litter . . . . .	5.5	9.7
Percentage mortality during first seven days due to natural causes . . . . .	80 per cent	2.4 per cent
No. killed in reducing litters to 7 young . . . . .	2	80
Nursing at 7 days:		
Number . . . . .	26	176
Av. weight . . . . .	9.2 gm.	14.5 gm.
Nursing at 21 days:		
Number . . . . .	24	176
Av. weight . . . . .	24 gm.	40.5 gm.

\* 4 of the original 19 were killed at 4 mos. because their weights were less than 200 gm. Only 2 of the Diet B group reached this weight at this age.

† Born and reared to 28 days of age on Diet BMS.

The infant mortality of the young observed alive on Diet BMS was 2.4 per cent during the first 7 days, whereas that in the case of Sherman Diet B was 80.0 per cent. However, there was no mortality on the former and only 2 deaths on the latter diet between 7 and 21 days. Reduction of litters to 7 young during the first week of life was practiced in both groups. The young of Diet BMS were considered to be of satisfactory weight for experimental work, averaging 40.6 gm. at 3 weeks, and they were in a healthy, vigorous condition. Those of Diet B were not heavy enough for experimental use and they did not have the healthy appearance of the animals of the meat scrap group.

#### *The Growth of Young on Sherman Diet B After Weaning*

During the time that the feeding of raw beef was practiced, the assumption was made that the meat improved lactation and that consequently it might not be necessary to supply it during the growth period

from weaning until the mating age, 120 days. As a test of this assumption 16 males and 22 females, from parents which had received raw beef, were weaned at 28 days (May, 1927) and placed upon Sherman Diet B without the meat supplement. After 11 days on this feeding régime, they began to lose weight and to show nutritional disturbances and, from 11 to 21 days after weaning, 29 died. The condition resembled closely that of the young which were placed on Diet B in July, 1926, when the colony was started. The survivors were given raw beef and an immediate improvement in condition and a resumption of growth took place.

On the other hand the animals used in Experiment 1, started in September, 1927, and those of Experiment 2, started in January, 1930, grew to the mating age on the Sherman Diet B and did not show the nutritional disturbances and cessation of growth noted above. Unless there is a seasonal influence, no reason is apparent as to why the different responses occurred.

#### *The Protein, Calcium and Phosphorus Contents of the Rations*

The inclusion of a meat scrap in Diet B caused an increase in the protein content from 19.00 per cent (Diet B) to 22.02 per cent (Diet BMS). Likewise there was an increase in calcium from 0.33 per cent to 1.19 per cent and of phosphorus from 0.49 per cent to 0.89 per cent.

The Ca:P ratio for Diet B used in this laboratory is 0.67 and that for Diet BMS 1.34. Smith and Bing (5) report 0.65 as the Ca:P ratio for Diet B and 1.16 as the ratio when one-half of the sodium chloride is replaced by calcium carbonate.

#### *Discussion of Results*

In Tables II and IV reproduction and growth records on Diets BM, BMS, and B are compared. All of the females reported in Table II were not mated the same number of times and therefore the fact that the number of litters per female is smallest in the case of Diet B is not due wholly to poorer reproduction. A detailed study of the colony records show the percentage of fertile females on Diet B to be about the same as on the other diets, yet the pregnancies are fewer when equal mating opportunity is afforded. The mortality of young from natural causes during the first week of life is markedly high in the case of Diet B. The average weights of young at 7 and 21 days on Diets BM and BMS are practically the same, and higher than those of Diet B. In Table II the average weights at these ages were calculated from much larger numbers of Diet BM and Diet BMS animals but in Table III the numbers are nearer the same order, and the same degree of difference in average weight prevails. The small number of



animals available on Diet B for weighing at 7 and 21 days is due to the smaller number observed alive per litter at birth, as compared with the other diets and to the heavy mortality during the first week of life.

The growth of male animals is summarized in Table IV. Those which received the raw beef supplement had been born and reared on this same dietary régime, whereas those on Diet BMS and Sherman Diet B were born and reared to 28 days of age on the former diet. As in the case of the females, Tables II and III, the rate of growth to the mating age on Diet B was less than that on the other two diets.

TABLE IV  
GROWTH OF MALES ON DIETS BM, BMS AND B

Age	Diet BM		Diet BMS		Diet B	
	No. of animals	Weight gm.	No. of animals	Weight gm.	No. of animals	Weight gm.
28 days.....	28	61	78	67.2	24	60.6*
120 days.....	25	292	78	300	17	259
9 mos.....	25	383	69	386	15	328

\* Born and reared to 28 days of age on Diet BM or BMS.

It is recognized that animals may not react favorably when changed from one diet to another but the change from Diet BMS to Diet B or from Diet BM to Diet B could scarcely be considered drastic. Certainly such a change would not be so drastic as that from a breeding diet to a vitamin-deficient diet. Furthermore, the change from one diet to another was not always accompanied by an immediate response. In the instance of the young breeding stock, reared on Diet BM and changed to Diet B, the failure did not manifest itself until after 11 days on Diet B. Hence poorer reproduction and growth on Diet B can scarcely be ascribed to a change of diet.

Table V presents a comparison of the growth performance of animals on Diet B as reported by Sherman and Campbell (1) with that of animals on the same diet modified by Macy, Outhouse, Long, and Graham (4), by Smith and Bing (5) and on Diets BM and BMS. Macy and associates use Diet B supplemented with fresh cabbage or lettuce and supply lactating animals with fresh cow's milk *ad libitum*. Smith and Bing replace one-half of the sodium chloride of Diet B with calcium carbonate and feed fresh lettuce daily. Lactating rats are supplied with 9 gm. of dried yeast per week.

TABLE V  
COMPARISON OF WEIGHTS OF ANIMALS RECEIVING DIETS BMS AND BM WITH THOSE OF OTHER COLONIES

Age	Sherman and Campbell (1)		Macy et al. (4)		Smith and Bing (5)				Diet BMS				Diet BM			
	Ave. wt., males	Ave. wt., females	Ave. wt., † males	Ave. wt., † females	No. of males	Ave. wt., males	No. of females	Ave. wt., females	No. of males	Ave. wt., males	No. of females	Ave. wt., females	No. of males	Ave. wt., males	No. of females	Ave. wt., females
Days	gm.	gm.	gm.	gm.		gm.		gm.		gm.		gm.		gm.		gm.
28	42.6	40.5	55	52	53	(27) †	11	(27)	78	67.2	159	62	28	61	47	55.6
120	247.1	182.1	286	192	52	330	21	219	78	300	156	198	25	292	45	196
235	—	—	370	230	29	(115)	12	(115)	60	379	96*	247	25	370	27*	256
300	314.9	222.2	(224)	(224)	—	—	—	—	47	399	114*	260	25	396	33*	268

† Values taken from curves.

‡ Numbers in parentheses indicate days of age at which weighing was made

\* Non-pregnant. Weights taken between pregnancies.

Each of the above modifications of Diet B has brought about an improvement in growth rate. Furthermore, our records displayed in Table II show a higher average weight for young at 7 and 21 days on Diets BM and BMS as compared with Diet B. At 28 days of age the animals of the colony of Macy and associates are slightly less in weight than those reported by Smith and Bing and those of this colony, but with the exception of the weights of females on Diets BM and BMS at 235 days their weights agree closely with those of our colony, and are lower than those reported by Smith and Bing. At 235 days, the females on Diets BM and BMS are slightly heavier than the others listed in Table V.

The reason for the improvement in growth which resulted from the several modifications of Diet B is not known. Smith and Bing state, "It is highly probable that the improved growth observed in our colony is due in large part to the more favorable relationship between calcium and phosphorus as well as to the alteration in the potential reaction of the diet." This tentative conclusion would not be supported by the results obtained in this colony when Diet B is supplemented with raw beef. In this instance there was no addition of calcium, the Ca:P ratio would probably be less than that of Diet B and the potential reaction of the diet would tend to become more acid. Diet B as modified by Smith and Bing became potentially more alkaline. The use of leaf tissue and of fresh milk with Diet B, as practiced by Macy and associates, would tend to increase slightly the Ca:P ratio and the calcium intake, but not as much as the addition of calcium carbonate in the amount used by Smith and Bing.

On the basis of the experience with this colony it would appear that raw beef and meat scrap have some property, not possessed by dry yeast, which improves the growth obtained with Diet B. Although Diet BMS contains 3.6 times as much calcium as Diet B, the improvement cannot be due chiefly to an increase in this element because raw beef causes essentially the same response. The greater growth rate obtained by Macy and associates, by Smith and Bing, and in this colony, may be due largely to a factor common to lettuce, cabbage, meat scrap, and raw beef. An investigation of the cause of the improvement in growth rate has been planned.

#### SUMMARY

1. The addition of fresh beef or of a meat scrap to a wheat-milk diet, used as a breeding colony ration for white rats, improved reproduction, and the growth rate and general vigor of the young as compared with the performance on the wheat-milk diet.

2. The use of dried yeast with the wheat-milk diet caused a slight improvement in reproduction but the growth rate and general vigor of the young were not bettered.

3. The improved performance on the wheat-milk diet supplemented with fresh beef or with meat scrap cannot be due chiefly to an increase in calcium and phosphorus or to an increase in the Ca:P ratio.

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# THE EFFECT OF RACHITOGENIC DIETS ON THE THYROID GLAND OF THE ALBINO RAT

BY JUANITA THOMPSON

*(From the Nutritional Research Laboratories of the Hospital for Sick Children  
and the Departments of Paediatrics and Pathology, University of Toronto, Toronto, Canada)*

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**P**ATHOLOGICAL changes associated with the feeding of rachitogenic diets have been described in the thyroid gland of dogs and rats. In 1921 E. and M. Mellanby (18) observed hypertrophy and hyperplasia in the thyroids of rachitic dogs. In the following year Murray (19) confirmed their observations, and, furthermore, pointed out that the degree of departure from the normal varied in proportion to the severity of the rickets. Although Davies (3) has not emphasized the fact, her findings, nevertheless, might be considered to cast doubt upon the latter conclusion. From her observations it would appear that greater variation in the thyroid gland occurred with the milder grades of rickets in puppies than with the marked. Sorour (22) noted in the thyroid of rats kept in darkness, and which had developed osteoporosis, a hyperplasia resembling Basedow's disease. In contrast to his results are those of McCarrison (12) who failed to produce goitre in rats kept in darkness fed on a deficient fat-soluble vitamin diet. Recently Krause and Munroe (7) have reported enlargement of the thyroid gland in rats on the Steenbock rachitogenic diet. The various authors have either incidentally mentioned the changes in the thyroid gland or have attributed them to some factor concomitant with the rachitic state. The difficulty of comparing the results obtained by the feeding of a variety of diets, the relatively few animals from which conclusions have been drawn, and the lack of unanimity of opinion, leads one to believe that relatively little is known concerning the relationship existing between these two conditions. Therefore, the present study was undertaken in the hope of demonstrating the factor involved in producing thyroid changes in animals fed on rachitogenic diets.

## EXPERIMENTAL

The opportunity to examine a large number of rachitic and non-rachitic rats kept under adequately controlled conditions was afforded by the extensive dietary studies carried on in the Nutritional Research Laboratories of the Sub-Department of Paediatrics, University of Toronto.

Since Sazaki (21), Burget (2), and McCarrison (13-14) have drawn attention to the frequency with which goitre arises in rats kept in an unhygienic environment, all the animals were confined in roomy wire cages with mesh bottoms. They were frequently washed and sterilized. No bedding was used. Usually fewer than five animals were placed in a cage.

The Steenbock rachitogenic diet, consisting of 76 parts of yellow corn, 20 parts wheat gluten, 1 part sodium chloride c.p., and 3 parts calcium carbonate c.p., and City of Toronto water, was employed either alone or with partial substitutions or additions of various ingredients. The experiments may be classified into three general groups, rachitogenic, antirachitogenic, and rachitogenic followed by antirachitogenic or the "line test." The animals were placed on the experimental diets at one month of age for approximately four weeks. The substances used in replacing in part yellow corn were non-irradiated whole wheat biscuit cereal,<sup>1</sup> the same cereal irradiated, plain bread, whole wheat, rolled oats, whole wheat or rolled oats ashed, chocolate, ashed chocolate, egg yolk or egg yolk ashed. Additions to the diets consisted of lard, shortening, viosterol or cod liver oil.

Aside from the routine experiments, a number of special tests were devised. To exclude the possibility of a vitamin deficiency other than D existing, the Steenbock rachitogenic diet was modified. Fourteen parts of wheat gluten and six parts of wheat germ were used in place of twenty parts of wheat gluten. One cc. of tomato juice per rat per day was added. Since it was considered that an inadequate amount of iodine either in the modified or the unmodified Steenbock diet might be a factor, potassium iodide was added to some. Two amounts were used—.00002 gram and .00128 gram per 100 grams of ration. By decreasing the calcium intake in relation to the phosphorus, even in the absence of the antirachitic vitamin, the condition has been considered to be less favorable for the development of rickets in rats (17). Therefore, in order to ascertain if an approximately direct quantitative variation existed between the changes in the thyroid gland and the rachitic phenomena, small groups of animals were placed on the Steenbock rachitogenic diet either with no added calcium or with  $\frac{1}{2}$ , 1,  $1\frac{1}{2}$  and 2 per cent, instead of the usual 3 per cent of calcium carbonate. Using the modified and the Steenbock rachitogenic diets either with or without iodine, a preventative series of experiments was carried out by supplying vitamin D in the form of either 1 per cent 1D Viosterol or  $\frac{1}{2}$  per cent cod liver oil. As much food and water as the animals would voluntarily consume were given.

<sup>1</sup> "Muffetts"

Control rats were those on the normal laboratory diet, as well as a group of wild rats.

The animals were started on these diets at one month of age and two were examined at weekly intervals beginning at the third week. The entire experimental period extended from December to June.

When the desired experimental period had elapsed the animals were etherized. Blood for phosphorus determination was collected by bleeding from the great vessels of the neck. Immediately following this procedure the thyroid glands together with the trachea and oesophagus were secured from two rats in each experimental group and placed in 10 per cent formalin. A representative number were x-rayed. The calcium content of the bones was later determined. After adequate fixation, paraffin sections were made and stained with hematoxylin and eosin. This routine varied in one instance in which the tissues from a group of thirty-two animals were not obtained until five hours had elapsed after death. During this interval the animals were kept in the refrigerator.

#### HISTOLOGY

The thyroid glands were classified according to their histological characteristics. Since transitions of one type into another were commonly encountered, it was often perplexing to arrive at a decision as to the particular group into which the specimen was to be placed. A conservative attitude was rigidly adhered to in all such doubtful instances.

Twelve animals one month old, intended to constitute the control group, were placed on the normal laboratory diet, which consisted of 64.5 per cent whole wheat, 15 per cent crude casein, 10 per cent whole milk powder, 8 per cent creamery butter, 1.5 per cent calcium carbonate, 1 per cent sodium chloride, meat and greens twice weekly, and City of Toronto water. One animal was killed weekly and the thyroid examined. For the first three weeks the glands grossly appeared small, and pale pinkish gray in color. Microscopically, the vesicles were small, regular in contour and fairly uniform. A scanty stroma of connective tissue divided them into irregular lobules. The larger vessels were readily demonstrable, but not dilated. Uniformly-staining, thin, colloid filled the acinar space. The lining epithelial cells were flat, having a small amount of acidophilic cytoplasm and an elongated or rounded, deeply-stained nucleus. From the fourth to the tenth week the glands increased in size and in depth of color. The isthmus became prominent. When studied microscopically a variation in the size and shape of the acini was noted. A few presented small plications. Usually the lobular divisions were incomplete. The vascular spaces showed a varying degree of engorgement. The colloid content was diminished and pale-staining, granular and largely vacuolated. The epithelial cells were increased in size, having acidophilic, granular cytoplasm, and enlarged, rounded, centrally placed, reticular nuclei, frequently surrounded by a clear space. Although these latter findings may merely represent physiological activity, rather than pathological change, nevertheless their inconsistency was considered to render them undesirable as a control group.

The difficulty of obtaining thyroid glands in the so-called resting state, led to the study of a series of 22 wild rats obtained from a stable situated in the centre of the city. These rats necessarily subsisted on a very mixed diet. In the gross, nineteen were found to have enlarged, grayish-red glands, while only three appeared of average size and pale, pinkish gray in color. Microscopically, the latter were made up of rather large vesicles which varied in size and shape. Lobular divisions were not readily discerned. No injection of the vessels was noted. The acini were well



filled by dense, granular colloid. The epithelial lining was of the low cuboidal variety, formed by cells having acidophilic non-granular cytoplasm and small, round, reticular or deeply stained nuclei. The former nineteen revealed acini varying markedly in size and shape and were frequently infolded. The majority had engorged vascular channels. The colloid content was depleted. Only pale-staining, granular, vacuolated remnants, partially filling the vesicular lumina, remained. The lining epithelium was of the tall cuboidal or columnar type, having achromatic, granular, occasionally vacuolated cytoplasm, and round or oval, centrally placed, reticular nuclei. Some of the vesicles were lined by more than a single layer of cells. Desquamation of a few cells into the acinar space was not an uncommon finding. Undifferentiated groups of epithelial cells were abundant. From these observations the conclusion drawn was that the thyroid glands of these wild rats showed changes to a degree not compatible with even the most marked variations of physiological activity, but rather the pathological manifestations of struma parenchymatosa diffusa.

Due to the difficulty in establishing a normal, the terms "non-hyperplastic" and "hyperplastic" were adopted in the classifying of the thyroid changes found in the experimental animals. Macroscopically, the non-hyperplastic glands were small and pale pinkish gray in color. Neither the isthmus nor the vessels were prominent. In contrast, the hyperplastic glands were of variable size but enlarged and were a deep grayish red in color. The isthmus was usually clear cut and the vessels outstanding.

The non-hyperplastic glands appeared to fall into two groups. The first group was composed of small and regular acini. The second group differed from the first in the variability of the size of the vesicles, the larger acini predominating. The acini, of both groups were divided into irregular lobules by a meagre stroma of connective tissue. The colloid content was either dense and bland or transparent, homogeneous or finely granular with vacuoles situated chiefly in the periphery. The epithelium lining the acini, containing the larger amount of colloid, was flat. The cytoplasm of the individual cells was small in amount, acidophilic and non-granular. The nuclei were elongated and intensely stained. On the other hand, the alveoli of the second group were lined by poorly staining cells of the low cuboidal variety, with rounded nuclei which stained less intensely than the elongated type. A clear halo frequently surrounded the nucleus. Small groups of undifferentiated epithelial cells, which probably represented collapsed or developing vesicles, were noted in most of the sections. Usually these appeared as syncytial masses having acidophilic cytoplasm. The vessels were not distended.

The hyperplastic glands were divisible into two major classes—those characterized by a predominance of small acini, and those composed essentially of large vesicles. Just as the non-hyperplastic groups had features in common, so had the hyperplastic groups, in that they presented varying degrees of epithelial hyperplasia, graded as slight, moderate or marked.

In the slightly hyperplastic group were placed glands in which the epithelial cells were somewhat larger than normal, having a greater amount of acidophilic granular cytoplasm, and usually showing a clear zone about the nucleus. The nuclei were enlarged, round or oval, pale-staining and reticular, situated more centrally within the cell. Colloid was present in the majority of the acini, but definitely decreased in amount, as indicated by its pale-staining character. Vacuolization was slight or moderate. A variable number of undifferentiated cell accumulations were noted. The vessels were moderately injected.

Glands presenting a moderate degree of hyperplasia were made up of vesicles which presented considerable variation in size and shape. Usually a few of the acinar walls showed plications. The colloid content was still further depleted, being absent in quite a proportion of the acini. That which remained had what might be termed a "washed out" appearance, in that it was pale-staining, thin, granular and largely vacuolated. The epithelial cells resembled those of the slightly hyperplastic groups, although occasionally they were larger. Not infrequently the lining cells were more than a single layer in thickness, often giving rise to small cellular buds protruding into the lumen. Lobular divisions were not discerned. Vascular injection was always present.

TABLE I  
EXPERIMENTAL ANIMALS ON RACHITOGENIC DIETS FOR FOUR WEEKS

Rachitogenic Diets	No of animals	X-Ray	Blood <sup>14</sup> phosphorus mgs/100 cc.	Ash content of bones %	Thyroid changes						Degenerative	
					Non-hyperplastic %	Hyperplastic %	Slight hyperplasia	Moderate hyperplasia	Marked hyperplasia			
Steenbock (a)	34	marked rickets	1.6	29.0	3	80	41%	30%	9%	17%		
(b), (g), (h)	52	marked rickets	2.1	36.4	0	100	29%	12%	9%	21%	9%	0%
							49%	42%	9%			
(c), (e)	4	marked rickets	1.4	29.9	100	0	32%	17%	15%	27%	6%	3%
(d), (f)	6	marked rickets	1.7	32.3	100	0						

The following rachitogenic diets were used:

(a) The unmodified Steenbock rachitogenic diet consisting of 76 per cent yellow corn, 20 per cent wheat gluten, 1 per cent sodium chloride c.p., 3 per cent calcium carbonate c.p.  
(b) The Steenbock rachitogenic diet modified by replacing 6 per cent of the wheat gluten by wheat germ and giving 1 cc. of tomato juice per rat per day.

(c) (a) plus .00002 grams of potassium iodide.

(d) (a) plus .00128 grams of potassium iodide.

(e) (b) plus .00002 grams of potassium iodide.

(f) (b) plus .00128 grams of potassium iodide.

(g) The Steenbock rachitogenic diet with yellow corn in part replaced by from 12½ to 37½ per cent white bread, or 33 per cent rolled oats, or 33 per cent whole wheat, or 33 per cent rolled oats plus the ash from 33 per cent whole wheat, or 30 per cent non-irradiated whole wheat biscuits, or the ash from 10 to 20 per cent egg yolk, or 2.2 per cent chocolate, or the ash from 2.2 per cent chocolate.

(h) The Steenbock rachitogenic diet plus 1 to 2 per cent shortening.

TABLE II  
EXPERIMENTAL ANIMALS ON ANTIRACHITOGENIC DIETS FOR FOUR WEEKS

Antirachitogenic diets	No. of animals	X-Ray	Blood <sup>24</sup> phosphorus mgs/100 cc.	Ash content of bones %	Non-hyper- plastic %	Hyper- plastic %	Thyroid Changes			
							Slight hyperplasia	Moderate hyperplasia	Marked hyperplasia	Degener- ative
							Small Large acini acini	Small Large acini acini	Small Large acini acini	
(a)	35	normal	3.8	44.2	0	51	17%	2%	32%	49%
							17%	2%	4% 28%	
(b)	8	normal	3.4	43.3	0	100	74%		26%	
							62% 12%		26%	
(c), (d)	4	normal	4.3	46.6	100	0				
(e), (f)	4	normal	4.5	46.2	100	0				
(g)	31	normal	3.1	44.9	32	68	56%	6%	6%	
							44% 12%	3% 3%	3% 3%	
(h)	12	normal	3.5	43.5	25	75	41%		34%	
							41%		17% 17%	

(i), (j)	28	normal	3.3	42.9	21	79	50%		3%	19%	7%
							50%	19%			
(k), (l)	4	normal	4.7	41.9	100	0			3%		
(m), (n)	4	normal	3.1	45.5	100	0					

The following antirachitogenic diets were used:

- (a) The Steenbock rachitogenic diet plus from  $\frac{1}{4}$  to 1 per cent 1D Viosterol (irradiated ergosterol).\*
- (b) The Steenbock rachitogenic diet with yellow corn in part substituted by 30 per cent irradiated whole wheat biscuits.
- (c) The Steenbock rachitogenic diet plus  $\frac{1}{4}$  per cent 1D Viosterol and .00002 grams of potassium iodide.
- (d) The Steenbock rachitogenic diet modified by replacing 6 per cent of the wheat gluten by wheat germ and giving 1 cc. of tomato juice per rat per day, plus  $\frac{1}{4}$  per cent 1D Viosterol and .00002 grams of potassium iodide.
- (e) The Steenbock rachitogenic diet plus  $\frac{1}{4}$  per cent 1D Viosterol and .00128 grams of potassium iodide.
- (f) The Steenbock rachitogenic diet modified as in (d) plus  $\frac{1}{4}$  per cent 1D Viosterol and .00128 grams of potassium iodide.
- (g) The Steenbock rachitogenic diet with yellow corn in part substituted by from 2 to 20 per cent egg yolk.
- (h) The Steenbock rachitogenic diet with yellow corn in part substituted by the ash from 10 to 20 per cent egg yolk plus  $\frac{1}{4}$  per cent cod liver oil.
- (i) The Steenbock rachitogenic diet modified as in (d) plus from  $\frac{1}{4}$  to 1 per cent cod liver oil.
- (j) The Steenbock rachitogenic diet plus from  $\frac{1}{4}$  to 1 per cent cod liver oil.
- (k) The Steenbock rachitogenic diet modified as in (d) plus  $\frac{1}{4}$  per cent cod liver oil and .00002 grams of potassium iodide.
- (l) The Steenbock rachitogenic diet plus  $\frac{1}{4}$  per cent cod liver oil and .00002 grams of potassium iodide.
- (m) The Steenbock rachitogenic diet modified as in (d) plus  $\frac{1}{4}$  per cent cod liver oil and .00128 grams of potassium iodide.
- (n) The Steenbock rachitogenic diet plus  $\frac{1}{4}$  per cent cod liver oil and .00128 grams of potassium iodide.
- \* 1D Viosterol was obtained by diluting 250D Viosterol with the requisite amount of corn oil.

The markedly hyperplastic group was composed of glands in which were the most striking changes. Again, a decided variability in the size and shape of the acini was noted. Infoldings as well as cellular buds were numerous. To observe any colloid content was unusual. The vesicular epithelium tended to be columnar. The cytoplasm of the cells varied in the staining qualities from section to section. The enlarged, round or oval nuclei usually occupied a central position within the cell. Many mitoses were seen. The vascular spaces were engorged.

As well as the two divisions above described, reference must be made to a third condition, which, for purposes of classification, has been termed the degenerative group. The glands placed in this category presented alveoli which were irregular in contour and from which the colloid had almost entirely disappeared. The epithelial elements showed profound changes. The cells were enlarged often to a marked degree, having acidophilic cytoplasm and small round or irregular or fragmented, pyknotic, centrally placed nuclei. They appeared either discretely, compactly or in heaped up masses. Desquamation into the lumen was commonly encountered. Engorgement of the vascular bed was usually observed.

### RESULTS OF STUDY

From the tables it will be seen that eighty-six experimental animals on the Steenbock rachitogenic diet, either unmodified or modified by the addition of vitamins other than vitamin D or with yellow corn in part substituted by a variety of substances, in 80 to 100 per cent, or, on the average, in 90 per cent of instances, showed a hyperplasia of the thyroid gland. This was slight in degree in 45 per cent, moderate in 36 per cent and marked in 9 per cent. Three per cent non-hyperplastic glands and 17 per cent showing degenerative changes were found in thirty-four animals on the unmodified Steenbock rachitogenic diet. In ten animals on the modified or unmodified Steenbock rachitogenic diet with added potassium iodide, the thyroid glands were non-hyperplastic.

Hyperplastic thyroid glands were found in an average of 75 per cent of forty-three animals on the Steenbock rachitogenic diet, unmodified or modified, with vitamin D added in the form of from  $1/4$  to 1 per cent 1D Viosterol, or with yellow corn in part substituted by irradiated whole wheat cereal biscuit. In 51 per cent of thirty-five animals on the Steenbock diet, with from  $1/4$  to 1 per cent 1D Viosterol added, thyroid hyperplasia was present. In 17 per cent of these it was slight, in 2 per cent moderate, and in 32 per cent marked. Forty-nine per cent of the thyroids of this group were placed in the degenerative class. In eight rats on either the modified Steenbock diet with  $1/2$  per cent 1D Viosterol added or with yellow corn replaced in part by irradiated whole wheat biscuits, 100 per cent had hyperplastic glands, the degree of which was slight in 74 per cent and marked in 26 per cent. On the Steenbock rachitogenic diet, modified or unmodified with  $1/2$  per cent 1D Viosterol to supply vitamin D, and potassium iodide added, eight animals had non-hyperplastic thyroid glands.

TABLE III  
EXPERIMENTAL ANIMALS ON THE STEENBOCK RACHITOGENTIC DIET FOR THREE WEEKS, FOLLOWED BY TEN DAYS ON VARIOUS TEST DIETS FOR THE PREVENTION OF RICKETS

Results of line test <sup>17</sup>	Number of animals	Blood <sup>28</sup> phosphorus mgs/100 cc.	Thyroid Changes						Degenerative		
			Non-hyperplastic %	Hyperplastic %	Slight hyperplasia		Moderate hyperplasia			Marked hyperplasia	
					Small acini	Large acini	Small acini	Large acini		Small acini	Large acini
1 plus healing	15	2.1	0	100	52%	40%	8%	8%			
										26%	20%
2 plus healing	33	2.4	9	91	48%	34%	9%	9%			
										30%	18%
3 plus healing	41	2.4	0	100	68%	26%	6%	6%			
										49%	19%
4 plus healing	31	3.4	0	100	45%	42%	13%	13%			
										32%	13%

TABLE IV  
EXPERIMENTAL ANIMALS ON THE STEENBOCK RACHITOGENIC DIET CONTAINING VARYING AMOUNTS OF CALCIUM CARBONATE

Steenbock rachitogenic Diet cont. CaCO <sub>3</sub>	No. of animals	Blood <sup>24</sup> phosphorus mgs./100 cc.	Ash content of bones %	X-Ray	Non-hyper- plastic %	Hyper- plastic %	Thyroid Changes				Degen- erative
							Slight hyperplasia	Moderate hyperplasia	Marked hyperplasia	Small Large acini acini	
							Small Large acini acini	Small Large acini acini	Small Large acini acini		
3%	34	1.6	29.0	marked rickets	3	80	41%	30%	9%	17%	
							29% 12%	9% 21%	9%		
1%	10	4.0	40.7	normal	0	100	20%	80%			
							10% 10%	60% 20%			
none	10	4.0	42.0	normal	0	100	56%	44%			
							45% 11%	44%			

When from 1/4 to 1 per cent cod liver oil, containing not more than .00125 to .005 mg. of iodine, was added per 100 grams to either the modified or the unmodified Steenbock diet, in twenty-eight animals non-hyperplastic glands were found in 21 per cent, and in 79 per cent hyperplastic glands. Of the latter 50 per cent were classified as having slight, 3 per cent moderate and 19 per cent marked changes. Seven per cent were of the degenerative type. Eight animals on similar rations with added potassium iodide had non-hyperplastic thyroid glands.

In thirty-one animals given the Steenbock diet, with from 10 to 20 per cent egg yolk replacing in part yellow corn, non-hyperplastic glands were obtained in 32 per cent, and 68 per cent were hyperplastic. Of these 56 per cent showed a slight grade of hyperplasia, and 6 per cent moderate, and 6 per cent marked degrees. When the ash from 10 to 20 per cent egg yolk was substituted and 1/2 per cent cod liver oil, containing approximately .0025 mg. of iodine, added to the Steenbock diet, in a group of twelve animals, non-hyperplastic glands were found in 25 per cent and hyperplastic glands in 75 per cent. Forty-one per cent of the latter organs presented slight changes and 34 per cent a marked degree of hyperplasia. These results indicated that a higher percentage of non-hyperplastic thyroid glands were obtained from animals whose diets contained egg yolk or the ash from egg yolk with added cod liver oil, than in other diets, except those to which potassium iodide had been added.

By combining the findings obtained at the end of the fourth experimental week on the Steenbock rachitogenic diet modified, unmodified, or with yellow corn substituted in part by a variety of substances, with or without added Viosterol, in 160 experimental animals, non-hyperplastic glands were present in 7 per cent, hyperplastic glands in 79.8 per cent and degenerative changes in 13.2 per cent. The degree of hyperplasia was slight in 47.4 per cent, moderate in 16 per cent and marked in 16.4 per cent. In 40 animals where cod liver oil replaced Viosterol in the diets, 23 per cent of the thyroid glands were non-hyperplastic and 73.5 per cent were hyperplastic. Hyperplasia was slight in 45.5 per cent, moderate in 1.5 per cent and marked in 26.5 per cent of these. Three and five-tenths per cent were of the degenerative type. With the addition of .00002 grams or .00128 grams of potassium iodide to the above diets non-hyperplastic thyroid glands were present in 100 per cent of twenty-six experimental animals.

One hundred and twenty experimental animals were placed for three weeks on the Steenbock rachitogenic diet, followed by ten days on various test diets for the prevention of rickets. These diets consisted of the Steenbock diet with 1/2 to 3 per cent 1D Viosterol, or 1/2 per cent shortening,



together with 1.5 per cent 1D Viosterol added, or with yellow corn in part substituted by 3.75 to 37.5 per cent white bread containing vitamin D.

Approximately 97 per cent of these animals had hyperplastic glands. The degree of hyperplasia was slight in 53 per cent, moderate in 35 per cent, and marked in 9 per cent. No regression in thyroid activity was demonstrable even with the greatest amount of bone healing.

When the above figures were combined with those previously indicated in the joint results obtained from the rachitic and antirachitic groups, exclusive of animals to whose diets cod liver oil or potassium iodide had been added, it was found that in two hundred and eighty experimental animals 4.6 per cent had non-hyperplastic thyroid glands, 88.4 per cent had hyperplastic organs, and 6.6 per cent of the glands were of the degenerative type. The degree of hyperplasia was slight in 50.2 per cent, moderate in 25.5 per cent and marked in 12.7 per cent.

The general nutrition of the animals was quite good. The average gain in weight of the animals on rachitogenic diets without additional iodine was 16 grams. When iodine was given the gain on the average was 20 grams. In animals on antirachitogenic diets, with no iodine supplemented, the gain was 24 grams. With additional iodine in antirachitic diets the gain was 31 grams. The greatest gain in weight, namely, 36 grams, was found in animals receiving egg yolk in their diet. Animals on the "line test" experiments gained least in weight. During the first three weeks very few showed any appreciable change. In the following ten-day period an average of 9 grams was gained.

The following observations were based on the study of one hundred and forty-six experimental animals, two from each group examined at weekly intervals. Hyperplastic changes in the thyroid glands began as early as the second experimental week and were consistently found to the end of the sixth week in animals placed at one month of age on the modified or the unmodified Steenbock rachitogenic diet, with or without added vitamin D, as 1/2 per cent 1D Viosterol. When .00002 gram of potassium iodide was added to the above diets the animals frequently showed slightly hyperplastic thyroid glands at the end of the third week, but from the fourth to the sixth week their glands were found to be non-hyperplastic. Non-hyperplastic thyroid glands were a constant finding in animals over a similar period to whose diets .00128 gram of potassium iodide had been added, instead of the smaller amount. Slightly hyperplastic glands were present in animals examined from the third to the seventh week when 1/2 per cent cod liver oil replaced 1/2 per cent 1D Viosterol in the diets. The thyroid glands from animals receiving .00002 or .00128

gram of potassium iodide in the cod liver oil containing diets were non-hyperplastic throughout a similar period.

Ten animals comprised a group on the Steenbock diet with 1/2 per cent calcium carbonate replacing the usual 3 per cent. At the end of four weeks all showed thyroid hyperplasia, the degree of which was slight in 20 per cent and moderate in 80 per cent. In smaller experimental groups receiving a diet in which from 1 to 2 per cent calcium carbonate was used, there was a gradual increase in the glands showing moderate hyperplasia. None of the animals receiving diets in which the calcium carbonate content was less than 3 per cent presented markedly hyperplastic glands.

In order to ascertain if the basal ration were capable of inducing thyroid hyperplasia merely due to its low iodine content, ten animals were placed on the Steenbock diet containing no calcium carbonate. After a month had elapsed all showed hyperplastic changes, the degree of which was slight in 56 per cent and moderate in 46 per cent.

The facts derived from the experimental results were that hyperplasia of the thyroid gland occurred in a high percentage of animals on the Steenbock rachitogenic diet with or without the addition of vitamin D in the form of Viosterol; that neither the supplying of an abundance of vitamins in the diets nor the replacement of a portion of the yellow corn by white bread, irradiated whole wheat cereal, rolled oats, whole wheat, rolled oats together with the ash from whole wheat, non-irradiated whole wheat cereal, egg yolk, the ash from egg yolk, chocolate, ashed chocolate, nor the addition of shortening to the diets, prevents its development; that approximately one-quarter of the experimental animals had non-hyperplastic glands when cod liver oil or an amount of egg yolk sufficient to prevent rickets, or the ash from egg yolk together with cod liver oil was added or substituted in the Steenbock rachitogenic diet; that no regression in hyperplasia was noted in animals despite a considerable amount of healing when test diets for the prevention of rickets were given for a ten-day period following the development of rickets; that the addition of .00002 gram or .00128 gram of potassium iodide to the Steenbock rachitogenic diet, either unmodified or modified, prevented thyroid hyperplasia at the end of four weeks; that .00002 gram of potassium iodide added to the modified or unmodified Steenbock diet was insufficient to prevent its occurrence entirely; that hyperplastic changes were frequently found in experimental animals having 1/2 per cent cod liver oil added to the Steenbock rachitogenic diet; that hyperplasia became manifest as early as the second week in animals receiving no additional iodine in their diets; that when the calcium carbonate content in the diet was reduced, a regression in the degree of

hyperplasia occurred; that hyperplasia occurred when no calcium carbonate was added to the Steenbock diet.

### DISCUSSION

Infections, intoxications, confinement, deficiencies, and excesses in food intake have been considered to influence the thyroid gland. Unrecognized agents, which in animals living in their natural environment have little effect, might exert powerful stimuli under experimental conditions. Although experimentally more than a single factor may be involved, only the most outstanding have been considered to be responsible for the manifestations. In view of our limited knowledge it would be futile to attempt to make finer distinctions at the present time.

Of the deficiencies affecting the thyroid gland the lack of iodine is well known. Most authors are agreed that in its absence pathological changes are evidenced in enlargement with hyperplasia. The results of partial deficiencies are less clearly defined. In their presence the effect of further stimulation from some other source would probably give rise to a greater response than when an adequate amount of iodine is available.

Hayden, Wenner and Rucker (4), and McClendon and Williams (16), produced thyroid enlargement in rats on diets having an iodine content of only 9 to 10 parts per billion. Tanabe (23) found thyroid hyperplasia in rats receiving a diet with low iodine content. In view of these observations, the finding of enlargement with hyperplasia in rats on the Steenbock rachitogenic diet was to be expected, since this diet obviously must have a low and variable iodine content, depending on the amount of the element in the soil where the grain has been grown (15). The giving of City of Toronto water, having an iodine content of 1.45 parts per billion (15), was insufficient to overcome this deficiency.

McCarrison (10) has stated that goitre in wild rats from different regions of India is excessively rare. For this reason, the finding of a high incidence of enlarged hyperplastic glands in the series of wild rats from this city was considered of interest. Toronto, Ontario, is situated in the endemic zone of the basin of the Great Lakes, where the existence of an iodine deficiency is well known (17).

Marine and Kimball (9) have shown that giving a small amount of any salt of iodine protects against simple goitre in lower animals and in man. McClendon and Williams (16), and Krause and Monroe (7) have found that the addition of iodine to diets low in this constituent produced smaller thyroid glands in rats. McCarrison (11) noted in rats and pigeons an increase in the colloid content of the thyroid when iodine was given with

a mixed diet. These observations receive further substantiation by the finding of non-hyperplastic thyroid glands in animals on diets capable of producing thyroid hyperplasia when supplemented by small amounts of potassium iodide.

According to the observations of E. and M. Mellanby (18), normal glands were found when cod liver oil was added to diets producing thyroid changes in dogs. Luce (8) and McCarrison (12-14) have reported the presence of normal glands in rats receiving different diets containing not more than 2.5 per cent cod liver oil. In the present study the amount of cod liver oil added was from one-quarter to one per cent. In animals receiving 1 per cent cod liver oil, non-hyperplastic and slightly hyperplastic glands were found, whereas those obtaining lesser amounts presented hyperplastic changes frequently to a marked degree. It would seem necessary, therefore, to add more than 1 per cent cod liver oil, containing approximately .000005 gram of iodine, to the Steenbock diet, if the production of the non-hyperplastic state is to be assured.

The occurrence of thyroid hyperplasia in dogs and rats on rachitic diets has been noted by a number of investigators, but has not been considered as the direct result of a deficiency of the antirachitic vitamin. However, that vitamins may play a part in this phenomenon has received consideration by McCarrison (12). He has been able to exclude vitamin D as a factor by the finding of goitre in rats receiving a sufficiency through exposure to direct sunlight. Krause and Monroe (7) obtained enlarged thyroid glands in rats after four weeks on the Steenbock rachitogenic diet. When ultra-violet radiation for the following four weeks was given, no decrease in size of the thyroid occurred unless potassium iodide was added to the diet. Their observations are in accord with the results of the present study, since enlargement with hyperplasia was found in experimental animals on the Steenbock rachitogenic diet or when vitamin D, in the form of Viosterol, was added to it. Hyperplastic changes were not shown by animals receiving sufficient iodine for their needs in the absence of the antirachitic vitamin.

Clinically, rickets, osteomalacia, and osteoporosis associated with goitre have been frequently reported in man and animals (6-9). The investigations of Parhon (20) and of Aub, Bauer, Heath and Ropes (1) indicated that the thyroid exerts an influence on the calcium metabolism, since they found that the calcium excretion could be greatly increased by means of the administration of thyroid extract. This observation received further substantiation by the finding of an excessive excretion of calcium in cases of hyperthyroidism and decalcification of bones in cases of long duration.

It has, furthermore, been shown that the laying down of calcium was decreased when the thyroid was removed and increased when thyroid was given. Although these studies suggest that there is a relationship, the manner in which this is effected is purely speculative.

Tanabe (23) found that the addition of 2 per cent calcium chloride enhanced the degree of hyperplasia occurring in rats on a low iodine-containing diet. While Hellwig (5) was unable to reproduce the slight hyperplastic changes on a similar basic ration, he found that hyperplasia was present when 2 per cent calcium chloride was added to it. The addition of 0.41 to 0.59 per cent calcium carbonate to a mixed diet increased the colloid storage in both rats and pigeons according to McCarrison (11). Supplementing iodine in high calcium diets, according to these investigators, produced relatively normal glands. Luce (8) noted the occurrence of normal thyroid glands in rats on diets containing 0.25 or 0.04 per cent calcium. Murray observed acute hyperplasia in the thyroid glands of dogs on rachitic diets, either calcium-free or with added calcium. Sorour (22), using diets containing 3 per cent calcium and .01 per cent iron, found hyperplasia of the thyroid glands of rats kept in darkness. The results suggest that hyperplasia will likely be produced when a diet of low iodine content contains as high an amount of calcium as 2 per cent. This deduction finds support in the initiation of thyroid hyperplasia in animals receiving the various modifications of the Steenbock rachitogenic diet without supplemental iodine all of which contained 3 per cent calcium carbonate.

The question of the relation between the amount of calcium in the diet and the degree of hyperplasia has to be considered. The results of study indicated that as the calcium carbonate content of the diet was decreased, a gradual diminution was apparent in the hyperplastic process. This transition may be further exemplified by comparing the degree of hyperplasia found when the Steenbock diet was given and when 1/2 per cent calcium carbonate replaced the customary 3 per cent. In the former instance 41 per cent were classified as slight, 30 per cent as moderate and 9 per cent marked hyperplasia, while in the latter 20 per cent showed slight changes and 80 per cent a moderate degree of hyperplasia.

In the present study thyroid hyperplasia in rachitic and non-rachitic rats was the result of an iodine deficiency, together with an excess of calcium in their diets. To secure non-hyperplastic glands there needs must be a favorable ratio between the iodine and the calcium in the intake. The presence of thyroid hyperplasia in rats on the Steenbock diet adds an avoidable complication which should be considered. In view of the fact that calcium is withdrawn from the bones in hyperthyroidism (1), and

although such a condition might actually favor the development of rickets, if one were testing various curative diets, a hyperfunctioning gland might actually deter the healing process.

The presence of degenerative changes in some of the glands adds a further complication. Although the highest incidence appeared in the only group which was not obtained immediately following exsanguination, to relegate these entirely to post mortem effects seems unjustifiable, since similar observations were also made in other groups. The explanation may be in part a greater tendency for the enlarging epithelial cells to shrink in fixative at a particular developmental period, as well as to become detached more easily when the vesicular content is less dense.

Variations in the degree of thyroid hyperplasia were always found within an experimental group. Why this should have occurred is difficult to explain. Such factors as animal susceptibility, season of the year, minor differences in the food from time to time (e.g., new grains, etc.), unrecognized intercurrent infections, the intake, the size of the animal, all exert an influence in any biological test. When one is using such a small experimental animal as the rat, very minute changes need must be considered. It is only when a particular observation has been made sufficiently often that a justifiable conclusion may be drawn from animal experimentation. For this reason, the present report, which is based on the study of a large number of animals, represents significant results.

In conclusion, I wish to thank Professor Alan Brown, Doctor F. F. Tisdall, and Doctor T. G. H. Drake for making available their experimental material for pathological study, and to express my gratitude to Miss Ruth Herbert for the excellent care taken of the animals as well as for the chemical determinations.

#### SUMMARY

1. The thyroid glands from 385 rats have been studied.
2. Diffuse hyperplasia was produced in rats through the feeding of the Steenbock rachitogenic diet or its various modifications.
3. Hyperplasia developed in the presence or absence of the antirachitic vitamin.
4. The factors underlying the development of hyperplasia in experimental animals on these diets were a deficiency of iodine, associated with an excessive amount of calcium carbonate.
5. Increasing the amount of calcium carbonate in diets deficient in iodine resulted in enhancement of the degree of hyperplasia.

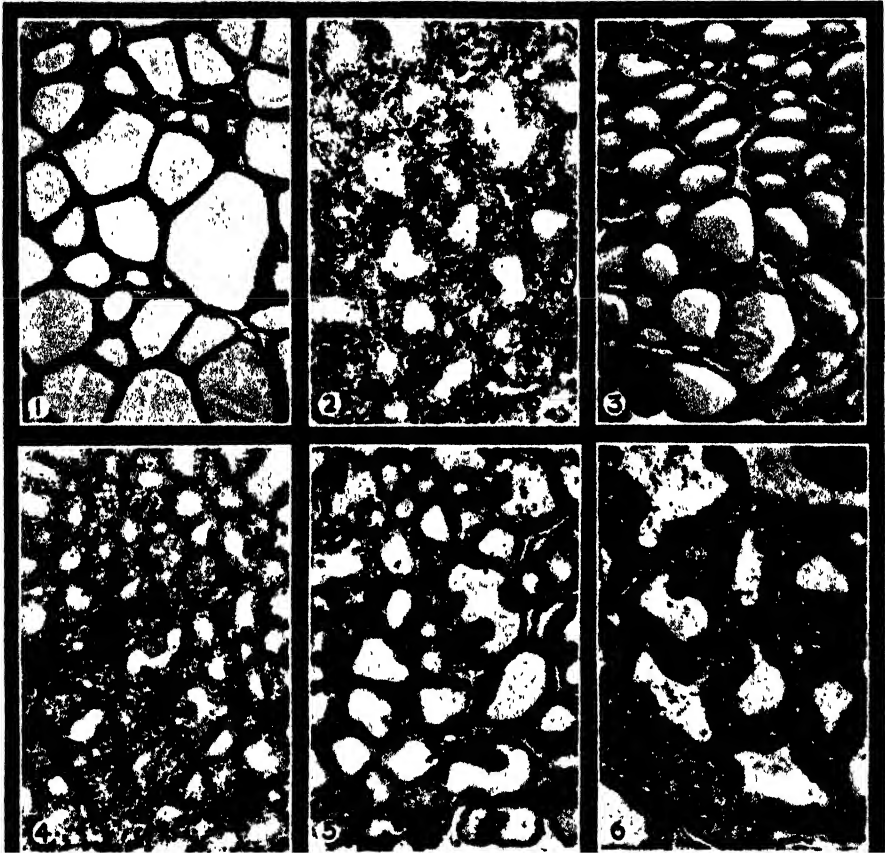
6. Variations in the degree of hyperplasia were found within similar experimental groups.

7. The addition of a small amount of potassium iodide to the diets prevented the development of hyperplasia.

8. A high incidence of goitre was found in a series of wild rats from this city.

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DESCRIPTION OF PLATE

- FIG. 1. Normal thyroid of untreated wild rat.  
FIG. 2. Idiopathic hyperplasia of thyroid in untreated wild rat.  
FIG. 3. Non-hyperplastic thyroid of rats receiving iodine.  
FIG. 4. Thyroid showing slight hyperplasia; low iodine, high calcium diet.  
FIG. 5. Thyroid showing moderate hyperplasia; low iodine, high calcium diet.  
FIG. 6. Thyroid showing marked hyperplasia, low iodine, high calcium diet.





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# THE UTILIZATION OF CALCIUM IN SOY BEAN DIETS

By

WM. H. ADOLPH AND SHEN-CHAO CHEN

*(From the Department of Chemistry, Yenching University, Peiping, China)*

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**I**N THE Occident a calcium deficiency is frequently found in diets regarded as adequate in other respects. In China the problem of an adequate supply of calcium is even more urgent, although a lack in calcium may also be accompanied by deficiencies in both protein and calorific intake. Cows' milk, upon which the people of western countries depend in large part for their calcium intake, does not figure seriously in the dietary of China. A dairy industry in China is essentially non-existent and at present is economically impossible. Moreover, the cost of raw milk, or of imported milk, either in tins or in the form of dried powder, is far beyond the reach of the average food purse. The dangers of calcium deficiency are somewhat mitigated by the fact that breast feeding is continued till the infant is three or four years old, but for the remainder of the growth period and for the entire period of adult maintenance the individual is dependent upon calcium derived from vegetable sources. A very considerable amount of this calcium is supplied by soy bean products. Soy bean curd (soy bean cheese) would seem to have filled, in part, the place which milk has occupied in the Occident. Recent years have seen an extended use of the closely related food product, soy bean milk, which has been successfully applied to infant feeding.

Unpublished data gathered in this laboratory indicate that the daily average calcium intake for the adult of 55 to 60 kilograms body weight in north China is 0.39 gram with a protein intake of 70 to 80 grams. The accepted figure for the minimum calcium requirement in the Occident is that of Sherman (1), 0.45 gram per day for an adult of 70 kilogram body weight, with a minimum protein requirement of 44 grams. To provide a margin of safety it has been suggested that the actual calcium intake should be between 0.7 and 1.0 gram per day. The average calcium intake of the adult in America is approximately 0.75 gram per day; the 0.39 gram calcium per day recorded for north China provides no such margin of safety.

Little is known regarding the degree to which the calcium of soy bean curd is utilized by the animal organism. Tso (2) has shown that calcium storage can be secured with infants fed soy bean milk. There is a certain

amount of evidence supporting the view that calcium proteinate is more easily absorbed than simple salts. But there is no general agreement on the extent to which vegetable calcium is assimilated by the body when compared, for example, with the calcium from milk. McClugage and Mendel (3) report that the calcium of spinach and carrots is poorly assimilated by dogs, and Boas (4) reported that spinach fed to rats was not favorable to calcium retention. Sherman and Hadley (5) found that in children the calcium of milk is more efficiently utilized than vegetable calcium. Blatherwick and Long (6) on the other hand, feeding cabbage, celery, and such vegetables, and Rose (7), experimenting with carrots, assert that calcium from vegetable sources is quite equal to the calcium of milk in the degree to which it is utilized in human adult maintenance.

The experiments here reported were planned for the purpose of determining the extent to which the adult can utilize the calcium of soy bean curd. Moreover, for the purpose of making a comparison between the effect of high and low protein intake on calcium utilization, two experiments were carried out. In one of these experiments, the protein intake was fixed at the more luxurious level of 65 to 90 grams per day. In the other experiment, the daily intake was 35 to 45 grams of protein, which, while admittedly low, provides a typical example of restricted protein intake, such as is not infrequently met with in the Orient.

#### EXPERIMENTAL

Three normal adults, Chinese, ages 18 to 27, ingested a typical north China diet so arranged that about 80 per cent of the calcium was furnished by soy bean curd. In a corresponding experimental period using the same diet, soy bean curd was replaced by milk as the source of calcium. Two of the subjects W.B.C. and T.L.W., both men, were laboratory technicians; the third S.C.C., a woman, was a member of the laboratory instructional staff. Two experiments were conducted at an interval of three months. Experiment 1 provided for a low protein intake; Experiment 2 provided a more liberal protein intake. The calcium intake in both experiments was set at 0.45 gram per day, this figure approximating the minimum calcium requirement, for it was presumed that differences in calcium availability would be more evident at a level approaching this value. Each experiment consisted of two experimental periods of seven days each, one following immediately upon the other; one period involved the ingestion of soy bean curd and the other the ingestion of milk as the principle source of calcium. The order of these two periods was reversed in the two experiments.

The foods selected for consumption were in general characteristic of the

Chinese diet, except that with all three subjects the consumption of milk was a distinct innovation. The milk was brought to the boiling temperature and ingested warm. The soy bean curd was sometimes cooked with cabbage and sometimes fried lightly in oil. Cornstarch and sugar are not common constituents of the diet, but small amounts were added in these experiments to increase the fuel value of the daily ration.

The food was prepared and eaten in the laboratory. The soy bean curd used was the variety manufactured by coagulation with salt bittern, the so-called northern bean curd, and was purchased from a local soy bean shop. The cows' milk was supplied by the local dairy. The soy bean curd and cows' milk were delivered daily. The bread was the typical steamed bread of north China made from white flour. The other foods were such as could be purchased in quantity and kept for the length of the experiment. The amounts of food consumed daily throughout the seven days of each period are recorded in Table I. The total calorific value of the diet is

TABLE I  
DAILY FOOD INTAKE—WEIGHTS IN GRAMS

Subject	Low Protein Diet				High Protein Diet			
	Milk		Soybean Curd		Milk		Soybean Curd	
	S.C.C.	W.B.C. T.L.W.	S.C.C.	W.B.C. T.L.W.	S.C.C.	W.B.C. T.L.W.	S.C.C.	W.B.C. T.L.W.
Food materials								
Milk . . . . .	300	300	—	—	290	290	—	—
Soy bean curd . . . . .	—	—	230	230	—	—	210	210
Rice . . . . .	140	140	140	140	280	110	320	110
Bread . . . . .	80	80	80	80	70	600	20	600
Meat . . . . .	50	50	50	50	150	100	100	100
Fat . . . . .	50	50	50	50	20	20	20	20
Cabbage . . . . .	100	100	100	100	50	—	50	—
Cornstarch . . . . .	—	55	—	55	—	36	—	31
Sugar . . . . .	50	95	50	95	20	35	10	30
Total calories . . . . .	1833	2233	1778	2178	2142	2845	1897	2744
Total protein . . . . .	36.76	36.76	44.00	44.00	62.24	83.83	61.63	92.15
Total phosphorus . . . . .	.665	.665	.616	.616	1.040	.822	.888	.793
Total calcium . . . . .	.448	.448	.449	.449	.443	.456	.442	.456
Per cent calcium furnished by:								
Milk . . . . .	80.3	80.3			78.5	76.3		
Soy bean curd . . . . .			80.9	80.9			78.5	76.3

calculated in the usual way. As a matter of interest, the total phosphorus content calculated from standard analyses is included also, although the experiment was directed solely to the question of calcium metabolism. The subjects ate three meals a day; the milk and soy bean curd in their respective periods were consumed, the milk at breakfast, the bean curd at the noon and evening meals. Distilled water only was used for drinking and in preparation of the food. No tea was drunk during the period of the experiment.

Calcium and nitrogen analyses were carried out on the daily samples of milk and bean curd and on each new lot of the other food materials as purchased; the usual care was taken in securing uniform samples. The averages of these analyses are indicated in Table II.

TABLE II  
AVERAGE NITROGEN AND CALCIUM CONTENT OF FOOD MATERIALS CONSUMED  
(in percentage of edible portion)

	Nitrogen	Calcium
Milk.....	0.528	0.120
Soy bean curd.....	1.28	0.162
Rice.....	1.28	0.018
Bread.....	1.32	0.013
Meat.....	2.56	0.007
Cabbage.....	0.17	0.049

Urine and feces were collected for the last four days of each 7-day metabolism period. The urine was collected and analyzed in 24-hour samples. The feces were collected as a composite for the four days, thoroughly mixed, and aliquots taken for analysis. Charcoal was used in marking off the feces for the different periods. The calcium in food, feces, and urine was determined by McCrudden's method (8). Nitrogen in the food and feces was determined by the Kjeldahl method; for nitrogen in urine, the micro-method of Folin and Denis (9) was employed.

## RESULTS

Tables III and IV give the calcium and nitrogen balances for the two experiments. In Table V, the balances are summarized in milligrams per day per kilogram of body weight. The low protein diet furnished an amount of protein which was quite insufficient for these subjects and negative nitrogen balances resulted.

TABLE III  
NITROGEN AND CALCIUM BALANCES  
First Experiment—Low Protein Diet  
(in grams per four-day period.)

Subject weight	Diet	Nitrogen				Calcium			
		Intake	Urine	Feces	Balance	Intake	Urine	Feces	Balance
S.C.C. 55.2 kilos	Milk	23.52	31.28	2.91	-10.67	1.792	0.395	2.120	-0.723
	Soy bean curd	28.16	28.48	3.42	-3.74	1.796	0.348	1.986	-0.538
W.B.C. 54.0 kilos	Milk	23.52	33.30	3.46	-13.24	1.792	0.419	2.380	-1.007
	Soy bean curd	28.16	32.32	7.46	-11.62	1.796	0.516	1.838	-0.558
T.L.W. 50.0 kilos	Milk	23.52	31.30	4.23	-12.01	1.792	0.373	1.658	-0.239
	Soy bean curd	28.16	27.84	4.85	-4.53	1.796	0.469	1.496	-0.169

TABLE IV  
NITROGEN AND CALCIUM BALANCES  
Second Experiment—High Protein Diet.  
(in grams per four-day period.)

Subject weight	Diet	pH of Urine	Nitrogen				Calcium			
			Intake	Urine	Feces	Balance	Intake	Urine	Feces	Balance
S.C.C. 55.0 kilos	Soy bean curd	6.0	40.44	40.24	5.00	-4.80	1.768	0.548	1.288	-0.068
	Milk	6.1	39.83	41.65	3.87	-5.69	1.772	0.508	1.211	+0.053
W.B.C. 55.7 kilos	Soy bean curd	6.0	58.96	42.40	6.84	+9.72	1.824	0.368	1.206	+0.250
	Milk	6.0	53.64	45.40	5.62	+2.62	1.824	0.381	1.104	+0.339
T.L.W. 55.0 kilos	Soy bean curd	6.3	58.96	45.62	6.41	+6.93	1.824	0.463	1.389	-0.028
	Milk	6.4	53.64	44.17	6.29	+3.18	1.824	0.382	1.597	-0.155

The subjects in the experiments were not accustomed to consuming such a large quantity of milk, somewhat over half a pint per day! They found the milk diet somewhat constipating, while the soy bean diet just as distinctively possessed the laxative properties of the usual Chinese diet.

TABLE V  
NITROGEN AND CALCIUM BALANCES  
(in milligrams per day per kilogram of body weight)

Diet	Nitrogen balance		Calcium balance	
	Milk	Soy bean	Milk	Soy bean
<b>Exp. 1: Low protein intake</b>				
Subject:				
S.C.C.....	-48.0	-17.0	-3.3	-2.5
W.B.C.....	-61.0	-54.0	-4.7	-2.6
T.L.W.....	-60.0	-23.0	-1.2	-0.8
Average:			-3.1	-2.0
<b>Exp. 2: High protein intake</b>				
Subject:				
S.C.C.....	-26.0	-22.0	+0.2	-0.3
W.B.C.....	+12.0	+44.0	+1.5	+1.1
T.L.W.....	+14.0	+31.0	-0.7	-0.1
Average:			+0.3	+0.2

Examination of the calcium balance figures indicates that there was no significant difference in the extent to which the calcium furnished by cows' milk and soy bean curd was assimilated. As to the amount of calcium ingested, it would seem that 0.45 gram per day is the very minimum possible on the type of diets here employed. In the second experiment, a more favorable calcium balance accompanied a liberal protein intake.

The results in these experiments are not complicated by great differences in the acid-base balance of the milk and soy bean curd diets. Computing the acid-base residues from the figures of Sherman and Gettler (10) for milk, and from our own for soy bean curd; it was found that the soy bean curd was slightly more basic than milk, to an amount equivalent to 0.5 cc. of normal base per 100 grams of the food material. This difference is too slight to cause any material variation in the retention of calcium in the two periods. As a check upon the acid-base balance, the pH of the urine of all three subjects during the second experiment, was determined colorimetrically each day. The pH of each of the subjects remained practically constant for the four days; the average values are shown in Table IV.

In experimenting with Chinese diets, the factor of bulk is usually a pronounced one. The diets consumed in these experiments were not characterized by excessive bulk, since it was necessary to avoid the usual large amounts of calcium-rich vegetables, such as cabbage, turnips, etc. The effect of bulk in the Chinese dietary on the assimilation of calcium remains to be studied.

#### SUMMARY

Metabolism experiments were conducted for the purpose of comparing the utilization by adult man of the calcium furnished by cows' milk and soy bean curd (soy bean cheese).

The calcium balances indicate that cows' milk and soy bean curd are equally effective as sources of calcium in the Chinese diet.

Increasing the protein intake facilitated the attainment of calcium equilibrium.

It is shown that a figure approaching 0.45 gram of calcium per day may be regarded as the minimum daily requirement for the Chinese adult of 55 kilograms body weight.

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# THE HEAT PRODUCTION OF CATTLE IN A RESPIRATION CALORIMETER AS RELATED TO THE RATE OF VENTILATION AND TO THE MOISTURE CONTENT OF THE AIR

By

E. B. FORBES, WINFRED W. BRAMAN, AND MAX KRISS\*

*(From the Institute of Animal Nutrition, Pennsylvania State College, State College, Pennsylvania)*

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THE subject of this discussion is significant in relation to studies of the heat production of cattle as the rate of ventilation and the moisture content of the ingoing air of the respiration chamber, or calorimeter, affect the moisture and carbon dioxide contents of the air within the apparatus, also the proportionate heat outgo by radiation and as latent heat of water vapor, and, finally, the total heat outgo from the animal body.

This is a matter of especial concern in indirect calorimetry, by the respiratory quotient procedure, since in the use of this method it is desirable so to restrict the rate of ventilation that the carbon dioxide and the oxygen contents of the outcoming air may differ as much as practicable from these measurements of the ingoing air—in order that the elements of error in analytical work may be acceptably small in comparison with the observations sought. The results of this study are also of interest because of their implications in relation to prevailing standards of barn ventilation.

The plan of this experiment, as outlined in Table I, provided for two series of four one-day heat measurements each, on a dry cow as the experimental subject. The ration was the same, throughout, and provided three-fourths of the maintenance requirement of energy. The rate of ventilation was alternately fast and slow on successive days in both series of observations; and the greater part of the moisture was frozen out of the incoming air during the second of the two series of heat measurements.

The slower rate of ventilation was approximately 15 per cent of the rapid rate, which latter was the rate ordinarily employed in direct calorimetric experimentation with cattle at this Institute.

In both series of observations intermediate periods of eight hours each, during which the rates of ventilation were maintained as in the observa-

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\* With the collaboration of Alex Black, Donald E. H. Frear, O. J. Kahlenberg, R. C. Miller, F. J. McClure, LeRoy Voria, and R. W. Swift.

tion periods to follow, were interposed between intervals of heat measurement; hence the experimental days do not coincide with calendar days.

TABLE I  
SCHEDULE OF EXPERIMENTATION

Period No.	Treatment	Intervals of Experimentation	Day of observation	Ventilation per hour
1	Air contained natural moisture	12 hours prel.		Liters
		24 hours heat meas.	1	35,120
		8 hours intermed.		
		24 hours heat meas.	2	5,454
		8 hours intermed.		
		24 hours heat meas.	3	35,656
		8 hours intermed.		
		24 hours heat meas.	4	5,449
2	Air freed from moisture by freezing	12 hours prel.		
		24 hours heat meas.	1	35,096
		8 hours intermed.		
		24 hours heat meas.	2	5,414
		8 hours intermed.		
		24 hours heat meas.	3	35,066
		8 hours intermed.		
		24 hours heat meas.	4	5,419

The purpose of these intermediate intervals was to establish constant conditions—of the animal, of the air in the chamber, and of the chamber itself—representative of the rate of ventilation to follow.

The animal was fed twice in each twenty-four hour measurement interval, the feedings being twelve hours apart; and the last feeding before the beginning of these twenty-four hour heat measurement periods took place four, eight, or twelve hours previously—on the different days. It seems unlikely, therefore, that the heat-stimulating effect of feeding, and of the early hours of digestion, could have contributed an uneven influence of important magnitude in the measurements of heat production.

The daily ration consisted—in terms of dry matter—of 1744 grams of alfalfa hay and 1821 grams of grain, the latter being a mixture devised for another purpose and containing 23.53 per cent wheat germ, 17.65 per cent of distiller's grains, 23.53 per cent corn meal, 17.65 per cent ground oats, 11.76 per cent ground barley, and 5.88 per cent linseed meal.

The water consumption of the cow during the first four-day period was 18.055, 15.055, 17.985, and 13.605 kgm. on successive days, and during the second four-day period was 12.920, 13.575, 15.730 and 9.495 kgm.,

likewise on successive days. The cow was watered twice daily, but inasmuch as the quantity consumed naturally varied much, from day to day, the foregoing weights of water drunk are not closely significant in relation to the differences in treatment.

The average weight of the cow in period 1 was 450.2 kgm. and in period 2 was 449.9 kgm.

The temperature of the air in the calorimeter was maintained constant within 0.2 degree Centigrade, the average being 21.8°.

In Table II are given the data for heat emission, at the different rates of ventilation, in relation to the moisture of the air and to the water vaporized,—the heat being measured by direct calorimetry.

In period 1 the relative humidity of the ingoing air naturally varied with the weather, and, on the average, 85 per cent of this moisture was frozen out during period 2.

The relative humidity of the air in the chamber varied, as would be expected, with the rate of ventilation, and the air which was dried by freezing did not carry out of the chamber quite as much moisture as did that which entered the calorimeter with its natural moisture content.

The data for latent heat of the water vapor measured as such (third column of figures) show that the dried air, and the rapidly circulating air, took up a greater quantity of water than did the humid and slow moving air.

In order to obtain measures of the total heat carried away from the animal as latent heat of water vapor, the latent heat of the water measured as vapor was added to the heat of condensation of the water which formed on the cooling system of the calorimeter.

These data for latent heat of total water (fifth column of figures) naturally varied from high to low values directly as the rate of ventilation.

The two columns of figures at the right of Table II give the percentages of the total heat emission eliminated as latent heat of water vapor, with and without corrections for the quantities of heat liberated on condensation of water on the cooling system of the calorimeter, the data including this correction being given in the right-hand column of the table.

These data show that approximately 40 per cent of the heat production left the animal as latent heat of water vapor, and, in general (one exception), that with decreased rate of ventilation there was decrease in the percentage of heat leaving the body as latent heat of water vapor.

In Table III are presented the data relating the rates of ventilation to the heat production, the rapid rate being such as to give, in the outcoming air, an average CO<sub>2</sub> content of 0.304 per cent, and the slow rate an average

TABLE II  
RELATIONS OF THE HEAT PRODUCTION OF A COW TO THE MOISTURE OF THE AIR AND TO WATER VAPORIZED

Treatment	Day of observation	Rate of ventilation	Rel. humid. ingoing air	Rel. humid. air in chamber	Latent heat of water vapor	Heat of condensation of water	Latent heat of total water	Total heat emission	Latent heat of water vapor; per cent of total heat	
									Not corrected for cond. of water	Corrected for cond. of water
Period 1: Air contained natural moisture	1	Rapid	23.7	68.4	Cals. 4180	Cals. 772	Cals. 4952	Cals. 12,447	33.6	39.8
	2	Slow	18.4	87.3	1000	3571	4571	11,420	8.8	40.0
	3	Rapid	21.6	66.3	4254	694	4948	11,735	36.3	42.2
	4	Slow	42.2	90.0	693	3807	4500	11,495	6.0	39.1
Period 2: Air freed from moisture by freezing	1	Rapid	3.7	54.7	4723	0	4723	11,524	41.1	41.1
	2	Slow	4.7	84.2	1142	3380	4521	11,709	9.8	38.7
	3	Rapid	3.2	58.9	5204	287	5491	11,919	43.8	46.1
	4	Slow	4.7	86.8	1177	3665	4842	11,937	9.9	40.6

TABLE III  
HEAT PRODUCTION OF A COW AS RELATED TO THE RATE OF VENTILATION IN A RESPIRATION CALORIMETER

Treatment	Day of observation	Rate of ventilation	CO <sub>2</sub> in outgoing air	Total heat production*	Time spent standing	Observed heat production corrected to standard day	Computed heat production corrected to standard day	Computed heat + observed heat
Period 1: Air contained natural moisture	1	Rapid	Per cent 0.312	Cals. 12,523	Hours 15.9	Cals. 12,242	Cals. 12,329	Per cent 100.7
	2	Slow	1.673	11,460	9.1	11,669	11,669	100.0
	3	Rapid	0.299	11,736	10.3	11,859	12,228	103.1
	4	Slow	1.711	11,435	8.9	11,657	11,799	101.2
Period 2: Air freed from moisture by freezing	1	Rapid	0.298	11,490	10.7	11,584	11,910	102.8
	2	Slow	1.763	11,643	14.2	11,485	11,695	101.8
	3	Rapid	0.307	11,905	13.6	11,790	12,113	102.7
	4	Slow	1.752	11,825	14.0	11,682	11,894	101.8

\* Corrected for heat stored in animal body.

CO<sub>2</sub> content of 1.725 per cent—the higher concentration being 5.67 times the lower. A CO<sub>2</sub> content of 1.0 per cent, in the outcoming air, is satisfactorily high for purposes of respiratory quotient calorimetry.

The total heat production as given in this table is derived from the heat emission, as stated in Table II, by correction for heat stored in the animal body. For the purpose of this study, however, it is necessary to compute, or to correct, the heat production to represent a standard day as to standing and lying, thus establishing a much more satisfactory basis of comparison of the treatments to which the subject was subjected.

This correction was made by employing the factor 16.0 Calories to represent the greater energy expense, per 100 kgm. live weight, of standing as compared with lying. This factor is the average of five determinations (15.1, 15.1, 16.9, 14.0 and 18.9 Cals.) with steers Nos. 47, 36, 57, 17, and 85, respectively, in experiments Nos. 238, (1) 240, (2) and 241 (3).

The heat production thus corrected is given in the second and third columns of figures from the right of this table, based in one case on the direct heat measurement and in the other on the heat as computed by indirect calorimetry.

In computing the heat production for period 1, by the indirect procedure, it was necessary to assume that the methane production for each day was the average of the values for daily methane production as determined in period 2, since methane was not determined in the first period. The justification for this procedure is that the same experimental subject and the same quantity of the same ration were involved.

The column at the right of Table III shows that the heat production was satisfactorily measured by both of the procedures employed, the maximum difference between the data for any one day's measurement by the two methods being 3.1 per cent.

This series of measurements of the heat production of a cow during eight successive days, separated by eight-hour intervals, would be considered as fairly satisfactory repeats if all represented the same conditions.

That the heat production of the first day was definitely higher than that of the second day could be plausibly explained as resulting from the fact that this was the first day that the cow had spent in the calorimeter; and it is common under these conditions for the heat production to be somewhat elevated; also, the animal changed position—as to standing and lying—more frequently on the first day than on any subsequent day.

From these data it is clear that the great differences which prevailed in the rate of ventilation produced no marked differences in the total heat production.

It is true, however, that with each change in the rate of ventilation from rapid to slow there was a slight decrease in the heat production; and, conversely, that with each change in the rate of ventilation from slow to rapid there was a slight increase in the heat production.

In view of the fact that the foregoing observation holds true in all of the six changes in the rate of ventilation, as indicated by both the direct and the indirect methods of measurement of the heat production, it is clear that with the slow rate of ventilation there is a slight but relatively unimportant decrease in the heat production. The writers suggest that the most likely cause of this effect is a decrease in the voluntary activity of the animal.

Attention is called to the facts that in the present experiment the ration was sufficient to supply only three-fourths of the maintenance requirement of energy; that the calorimeter chamber was maintained at a temperature of 21.8°C; and that the chamber was efficiently cooled by water circulating inside it.

With full feeding of the experimental subject the heat production would have been much higher; and in the use of respiration chambers in warm weather the temperature of the air might be higher than it was during this experiment. Under such circumstances, therefore, an adequate cooling device would probably be required, in connection with the respiration chamber, in order to prevent discomfort of the animal and disturbance of its heat production.

#### SUMMARY

The heat production of a dry cow, on a constant ration, was measured in a respiration calorimeter by direct and also by indirect calorimetry, in eight consecutive twenty-four hour intervals of observation, to determine the effects of the rate of ventilation of the calorimeter on the paths of outgo of heat from the animal and on the quantity of the total heat produced.

The rate of ventilation was rapid or slow in alternate twenty-four hour intervals, the rapid rate being about 35,000 liters per hour, and the slow rate from 5,414 to 5,454 liters per hour; and in the second four of the eight intervals of observation about 85 per cent of the moisture was removed from the ingoing air by freezing.

The carbon dioxide content of the outcoming air at the slow rate of ventilation was 10.3 times as great, and at the high rate of ventilation 1.8 times as great, as that permitted by King's standard (4) (.167 per cent) for barn ventilation.

The moisture content of the outcoming air at the slow rate of ventila-



tion was 1.4 times as great as at the rapid rate,—a large proportion of the moisture given off by the animal having been condensed on the cooling system of the calorimeter.

With both methods of determination of the heat production there was, with each change in the rate of ventilation from rapid to slow, a slight decrease in the heat production; and with each change from slow to rapid, a slight increase in the heat production.

Approximately 40 per cent of the heat produced was eliminated by the animal as latent heat of water vapor, the quantity of which generally decreased to a slight extent with decrease in the rate of ventilation.

Inasmuch as the carbon dioxide content of the outcoming air at the slow rate of ventilation averaged 1.725 per cent, whereas a carbon dioxide content of 1.0 per cent is ample for satisfactory respiratory quotient calorimetry, the authors conclude that it is unlikely that the rate of ventilation would affect the heat production in respiratory quotient calorimetry if the respiration chamber is equipped with an adequate cooling device.

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# THE ECONOMY OF CONVERSION OF FOOD ENERGY INTO MILK ENERGY BY THE DAIRY COW

By

E. B. FORBES AND LEROY VORIS

*(From the Institute of Animal Nutrition, Pennsylvania State College, State College, Pa.)*

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A SIGNIFICANT consideration in relation to milk production as a factor in systems of agriculture is the fundamental economy with which the cow transforms her own feed into food available for human beings—as measured by the proportions of the food energy which the cow turns into milk.

An opportunity to make observations on this matter having arisen in connection with a series of year-long mineral balance studies, the results of this incidental study are here separately presented.

One point of view from which this matter has already been studied is that of the relative economy of utilization of metabolizable energy for the maintenance, body increase, and milk production of cattle.

Thus, Forbes, Fries, Braman and Kriss (1) found the average rates of utilization of metabolizable energy for maintenance, lactation and body increase to be as 1.0 for maintenance, 0.985 for lactation, and 0.761 for body increase.

In the present study, however, this point of view is precluded by the fact that the plan of experimentation provided no basis for determination of the maintenance requirement of energy.

At the same time a broadly significant and practical consideration in this relation is that of the gross return of milk energy from a year's investment of food energy, without separation of the quotas for maintenance and actual production; and this is the point of view of the present study.

The results of this study, therefore, apply with exactness only to cows of the particular size and productive capacity of the subjects of this investigation; but inasmuch as these cows represented moderately high normals—well above the commercial average—the conclusions reached are representative of a rather definite grade of high-class milk production.

The plan of investigation, which is outlined in Table I, provided for the experimental feeding of 12 cows during an entire period of lactation and gestation, which is considered normally to extend over one calendar year; but on some unexplained account the cows did not conceive readily, and three individuals were not pregnant at the time the experiment was

terminated. The slight effect of pregnancy, therefore, in the gross economy of food utilization for milk production, was not present in full normal degree.

Since the main object of the investigation was a study of mineral metabolism, the discussion of the details regarding the experimental program, the animal subjects, and the methods of procedure will be given elsewhere. In brief, however, the conditions of experimentation were as follows:

The subjects were mature Holstein Friesian cows, either high-grade or pure-bred, which were known to be free from tuberculosis and from infectious abortion. As to state of flesh, they were rather uniformly in ordinary working condition at the beginning of the experiment. In the course of the year most of the cows gained in weight about as is usual with cows which are with calf, but No. 1 became sick and lost materially in weight; No. 12 did not quite maintain her weight; while No. 7 improved in condition quite materially.

The experiment began, for each cow, immediately after calving; it was necessary, therefore, to select cows due to calve at about the same time.

The cows received normal rations of hay, grain, and corn silage. Half of the cows received alfalfa hay and half of them timothy hay; and among these two groups two received bone meal, and two received limestone, as mineral supplements, while two received no mineral feeds except for common salt, which all received.

The cows were given approximately as much feed as they would eat without remainder—which signifies that they were virtually full-fed.

On account of the difference in the protein content of timothy and alfalfa hay the grain mixtures fed with the two kinds of hay were compounded in such manner as, in a measure, to compensate for this difference. These grain mixtures were composed of corn meal, ground oats, ground barley, distiller's grains, wheat germ, and soy bean or linseed oil meal. It was not possible, however, exactly to equalize the protein content of the rations, because the relative consumption of grain and roughage was neither constant nor exactly controllable. Also the protein content of the rations lacks critical significance since all of the cows were given more protein than was required—to provide against the possibility that a deficit in protein intake would unfavorably affect the utilization of mineral nutrients.

In the determination of the feeding schedule a prevailing rule of practice was kept in mind, in accord with which a cow is given one per cent of her live weight in hay, three per cent of her live weight in corn silage, and

one pound of grain for each three to three-and-a-half pounds of milk produced; but at no time was it possible to feed all of the cows on exactly this basis, or indeed on any other such schedule of allowance. In fact, in order to maintain the coöperation of the experimental subjects, and to have the rations entirely consumed, it was found necessary to respect the individual preferences and capacities of the cows, and in reality to allow each cow in large measure to determine her own feeding schedule. A continuing effort was made to keep feeding conditions comparable, but it was not possible to accomplish this purpose without compromise.

The obvious preferences of individual cows for particular kinds and quantities of feeding stuffs are not with certainty explicable, but contributing influences seem to be inherited limitations of capacity, the status of the nutritive reserves of the body as determined by previous feeding and production, the condition of the teeth, and the live-weight of the animal in relation to productive capacity.

The hay was fed cut into short lengths, for convenience in weighing, handling, and sampling for chemical analysis; and the grain was all fed ground.

The cows were fed three times per twenty-four hours during liberal milk production, and twice a day later in the period of lactation.

The animals were housed in an ordinary cow shed, with concrete floor and mangers, which was adapted to the experimental purpose by the installation of galvanized iron feed boxes, to prevent waste, and specially designed equipment for the collection of excreta.

In lieu of bedding the cows were provided with thick felt pads, covered with canvas, and nailed to plank overlays on top of the concrete stall floors.

The cows were brushed each day, and scrubbed with soap each week; also they were exercised each day by being led a certain distance—usually about a quarter or half of a mile.

The experimental periods were normally 28 days in length, and energy determinations were made in a bomb calorimeter by standard procedure.

Since there were but two animals on each experimental treatment, the evidence was quite insufficient to warrant the consideration of the effects of the feeding treatment on the economy of utilization of feed energy. It is proper, therefore, only to consider the results as a whole, as indicating in a general way the efficiency of cows to transform feed energy into milk energy (a) during complete periods of lactation, and (b) during a calendar year.

In averaging the data in Table II,<sup>7</sup> cows Nos. 2a and 2b, and 4a and 4b

TABLE I  
SCHEDULE OF EXPERIMENTATION

Cow No.	Treatments	Date of beginning	Days on exper.	Days milking dur. exper.	Initial age of cow	Initial functional status of cows	Final functional status of cows
1	Alfalfa hay, grain, silage	Dec. 15, 1929	365	317	4 yrs. 6 mo.	Immed. after calv.	Dry; farrow
2a*	Alfalfa hay, grain, silage	Dec. 16, 1929	123	123	5 yrs. 5 mo.	Immed. after calv.	123 days after calv. 18 days preg.
2b	Alfalfa hay, grain, silage	May 20, 1930	245	182	6 yrs. 9 mo.	120 days after calv.	Dry; 210 days preg.
3	Alfalfa hay, grain, silage, bonemeal	Dec. 10, 1929	292	292	6 yrs. 2 mo.	120 days after calv.	Aborted; 210 days preg.
4a**	Alfalfa hay, grain, silage, bonemeal	Dec. 12, 1929	98	98	7 yrs. 1 mo.	Immed. after calv.	98 days after calv.
4b	Alfalfa hay, grain, silage, bonemeal	May 21, 1930	241	168	5 yrs. 10 mo.	69 days after calv.	Day of calv.
5	Alfalfa hay, grain, silage, limestone	Jan. 11, 1930	365	303	5 yrs. 3 mo.	Immed. after calv.	Dry; 220 days preg.
6	Alfalfa hay, grain, silage, limestone	Dec. 13, 1929	365	317	7 yrs. 11 mo.	Immed. after calv.	Dry; farrow
7	Timothy hay, grain, silage	Dec. 1, 1929	365	310	10 yrs. 11 mo.	Immed. after calv.	Dry; 50 days before calv.
8	Timothy hay, grain, silage	Nov. 30, 1929	365	327	4 yrs. 11 mo.	Immed. after calv.	Dry; 100 days before calv.
9	Timothy hay, grain, silage, bone meal	Dec. 31, 1929	365	304	6 yrs. 9 mo.	Immed. after calv.	Dry; 216 days preg.
10	Timothy hay, grain, silage, bone meal	Dec. 29, 1929	365	305	7 yrs. 10 mo.	Immed. after calv.	Dry; 124 days preg.
11	Timothy hay, grain, silage, limestone	Dec. 20, 1929	365	320	5 yrs. 8 mo.	Dry, 43 days before calv.	Dry; 55 days before calv.
12	Timothy hay, grain, silage, limestone	Dec. 9, 1929	365	319	8 yrs. 9 mo.	Immed. after calv.	Dry; farrow

\* Removed from the experiment because of a tumor in the nasal passage.

\*\* Removed from the experiment because of an abscess on the heart, caused by penetration by a foreign body in the rumen.

TABLE II  
THE EFFICIENCY OF TRANSFORMATION OF FEED ENERGY INTO MILK ENERGY

Cow No.	Treatment	Days of lactation	Milk produced	Gross energy of feed during lactation	Energy of milk	Recovery of feed energy during lactation	Gross energy of feed during 365 days	Energy of milk divided by energy of feed for 365 days	Initial live weight	Final live weight (365 days)
			Kgm.	Cals.	Cals.	Per cent	Cals.	Per cent	Kgm.	Kgm.
1	Alfalfa hay, grain, silage	317	6,762.3	20,506,160	4,400,238	21.46	22,688,490	19.39	661	611
2a; 2b	Alfalfa hay, grain, silage	305*	4,422.6	15,728,240	3,084,259	19.61	18,418,965	16.75	489; 513	—
3	Alfalfa hay, grain, silage, bonemeal	292	4,808.7	15,820,334	3,147,747	19.90			579	—
4a; 4b	Alfalfa hay, grain, silage, bonemeal	266**	4,036.1	14,201,883	2,917,072	20.54			602; 456	—
5	Alfalfa hay, grain, silage, limestone	303	5,200.4	16,278,297	3,403,506	20.91	18,933,379	17.98	528	620
6	Alfalfa hay, grain, silage, limestone	317	5,847.1	18,007,650	3,954,107	21.96	19,826,079	19.94	526	564
7	Timothy hay, grain, silage	310	5,303.1	17,553,839	3,167,222	18.04	20,178,974	15.70	559	714
8	Timothy hay, grain, silage	327	5,438.4	18,541,678	3,511,764	18.94	20,087,435	17.48	497	590
9	Timothy hay, grain, silage, bonemeal	304	5,332.7	15,970,094	3,524,987	22.07	18,315,622	19.25	478	547
10	Timothy hay, grain, silage, bonemeal	305	4,208.8	14,421,704	2,963,628	20.55	16,571,279	17.88	435	497
11	Timothy hay, grain, silage, limestone	320	5,730.2	17,635,042	3,853,669	21.85	19,530,045	19.73	493	522
12	Timothy hay, grain, silage, limestone	319	4,380.2	14,022,000	3,273,567	23.35	15,503,340	21.12	425	415

\* Cow 2a, 123 days, and cow 2b, 182 days.

\*\* Cow 4a, 98 days, and cow 4b, 168 days.

were eliminated because in these cases two cows were used to cover the year; and No. 3 was eliminated because she did not finish the calendar year of experimentation. The results as determined for these cows, however, are not without value.

The averages of the data for the remaining nine cows are as follows: Live weight of cows, 520.8 kgm. (1146 lbs.); milk produced per year of lactation, 5,356 kgm. (11,783 lbs.); recovery of feed energy during lactation, 20.96 per cent; and recovery of feed energy during a calendar year, 18.68 per cent.

These rates of efficiency were obviously related to gain or loss of body weight. Thus, the three farrow cows, Nos. 1, 6 and 12, were characterized by relatively high rates of efficiency in transforming feed energy into milk energy; but the nearly equal performance of three other cows, Nos. 5, 9, and 11, which at the end of the year were advanced in pregnancy, indicates that under the existing conditions the energy requirements of gestation did not constitute a major influence as affecting efficiency of milk production.

The arrangement of the cows in the order of their gain in weight, however, incidentally arranges them, in general, in the reverse order of their efficiency to transform feed energy into milk energy. This generalization is most notably untrue in relation to one cow, No. 1, which was the best producer of all. This cow appears not to have been as efficient as implied by her failure to gain in weight, this departure from the general order being due, at least in part, to the fact that she became sick, and lost markedly in live weight during her twelfth month on experiment, apparently as a result of the death of her fetus about two-and-a-half months after conception; also this cow occasionally leaked milk from the udder, which was not accounted for, her true efficiency as a milk producer, therefore, being somewhat greater than as indicated by the quantity of milk with which she was credited.

The most efficient cow, No. 12, did not conceive during the experiment; she did not quite maintain her live weight; and she transformed 23.3 per cent of her feed energy into milk energy during 319 days of lactation (21.1 per cent during 365 days).

The least efficient cow, No. 7, gained 155 kgm. in live weight, produced a calf, and transformed 18.04 per cent of her food energy into milk energy during her 310 days of lactation (15.7 per cent during 365 days).

#### SUMMARY

Nine Holstein Friesian cows, with an average live weight of 520.8 kgm. (1146 lbs.), and an average milk production, for one period of lactation,

of 5.356 kgm. (11,783 lbs.), transformed 20.96 per cent of their feed energy into milk energy during a period of lactation averaging 313 days in length; and transformed 18.68 per cent of their feed energy into milk energy during a calendar year.

The most efficient cow converted 23.35 per cent of her feed energy, and the least efficient cow 18.04 per cent of her feed energy, into milk energy during the period of lactation.

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# A COMPARATIVE STUDY OF RABBITS MAINTAINED ON BARLEY OR ALFALFA

By

FRITZ BISCHOFF, W. D. SANSUM, M. LOUISA LONG,  
AND RICHARD D. EVANS

*(From The Chemical Laboratory of the Potter Metabolic Clinic,  
Santa Barbara Cottage Hospital, Santa Barbara, California)*

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## INTRODUCTION

IN EARLY diet experiments from this laboratory concerned with the production of renal and blood vessel changes, Nuzum, Osborne, and Sansum (1) concluded that "there also was evidence of acidosis, as shown by a continued decrease in the carbon dioxide of the blood plasma of the oat and liver protein groups of animals." In later experiments (2), the same liver diet (urine pH 6.2) for the same period was fed rabbits with spontaneous nephritis and the results compared with rabbits with spontaneous nephritis on a barley-alfalfa diet (urine pH 7.0 to 8.5). In spite of the kidney damage, the rabbits on the liver diet failed to show evidence of acidosis as judged by a comparison of the  $\text{CO}_2$  combining powers of the series on liver with the series on barley and alfalfa. The initial mean for the  $\text{CO}_2$  combining powers was 47.6 with a standard deviation of 2.7, eight samples being taken. At the termination of the diet régime, the mean for each series, with four in a series, was well within the standard deviation of the mean. Judging by the standard deviation of the mean established by the figures of Nuzum's latter series, the normal variation for his controls in the earlier paper was five times the standard deviation of the mean. Page (3) compared rabbits on an acid diet of oats and bread with rabbits on an alkaline diet consisting of cabbage, carrots and hay. The alkali reserve was determined weekly for a period of five weeks. The results for the acid diet are within once the standard deviation of the mean of the initial values of Nuzum's second series. The alkali reserve for the alkaline diet series was considerably higher even after one week of dieting. However, these rabbits developed a diarrhea and showed loss in weight in a week's time, so that the condition of alkalosis can hardly be ascribed to the alkaline ash of the diet. A similar experience in a human experiment of our series will be described later in another paper. The remarkable agreement between Nuzum's second initial series and Page's values after an acid diet is evidence against the production of a lowered alkali reserve in the rabbit by an acid ash diet.

The earlier work of Nuzum, Sansum, and Osborne indicated a production of acidosis in the rabbit by diet and suggested a possible change in the pH of the blood. The original purpose of the present experiments was to establish whether or not an acid diet was able to change the pH of the blood, using the newer refined methods of analysis.

### EXPERIMENTAL

*Diet régime.* Fourteen rabbits approximately six months of age were divided into two groups. In each group three rabbits were selected for the successive determinations of blood pH and CO<sub>2</sub> content, the blood to be drawn from the heart. The remaining rabbits were to be kept for substitution in case of accidental death due to injury to the heart, or, barring this accident, to serve as controls for the effect of continued heart puncture throughout the experiment. For two weeks both groups were placed on a mixed alfalfa-barley diet, the blood data being determined for the three selected rabbits in each group. The one group was then placed exclusively on alfalfa and the other group exclusively on barley for the next six months. During this period it was observed that the rabbits on the barley diet destroyed the wooden slats of their cages, making an attempt to eat the wood. New cages lined with metal were installed when this observation was made. Eating the wooden slats had no effect upon the acidity of the urine, so would not be a factor in the experiment. At any rate, the time interval during which the wood was eaten was a small fraction of the total period of the experiment. As originally planned, the experiment was to be terminated at this time, but, as no chemical changes in the blood could be detected, and as the rabbits had all survived, the regime was continued until all the animals on the barley diet were dead. From the sixth month on, the rabbits on the barley diet were given a small quantity (20 gm.) of alfalfa once a week. Food was withheld the night before the morning when the bleedings were made. The rabbits lived outdoors throughout the experiment, except when urine collections were made. The rabbits were then placed in metabolism cages and fed their barley or alfalfa *ad libitum*, a twenty-four hour specimen being collected. Two of the rabbits on the alfalfa diet were killed at the time the first rabbits on the barley diet died. The remainder of the alfalfa group was sacrificed two months after all the rabbits on the barley diet had died.

*Methods.* The samples of blood for pH and total CO<sub>2</sub> determination are valueless unless taken under basal conditions. In working with laboratory animals, this presents a problem of considerable difficulty and one which has in the past been entirely overlooked by many workers, so that other-

wise excellent results are worthless for interpretation. In the earlier work in this laboratory, blood for  $\text{CO}_2$  combining power was obtained by cutting the marginal ear vein of the rabbit and collecting the blood under oil. In order to facilitate the speed of bleeding, the blood vessels of the ear were either dilated by rubbing the ear with ether or by placing the ear over an electric light bulb. There are two serious objections to this procedure; (1) an unavoidable loss of  $\text{CO}_2$  due to the large volume of oil required and the long time required for bleeding, and (2) the uncertainty as to whether the blood was venous or arterial, since venous blood becomes virtually arterial with marked dilation of the vessels. To establish the possible error of this procedure samples of venous and arterial blood were taken from a rabbit's heart, a procedure which was successful after some practice, and both the pH and total  $\text{CO}_2$  content determined. The pH of the venous blood was 7.46, that of the arterial, 7.49. The total ( $\text{CO}_2$ ) of the venous blood plasma was 51.9 volumes per cent, that of the arterial blood 39.0 volumes per cent. The great variation in the  $\text{CO}_2$  combining powers of the control groups in Nuzum's first series, as compared with the second series, may possibly be accounted for by the early technic of blood sampling. The practice of stunning rats by a blow on the head and then bleeding from the heart is a common practice in some laboratories and gives erratic results due to the changes of respiration, leading to high or low results depending upon over- or under-ventilation. An example is cited. A rat was bled from the heart. He was then stunned by a blow on the head and again bled from the heart. The first plasma pH was 7.53, the second, 7.16. In the present studies the blood was drawn from the heart.

The pH of the urine of the rabbits on the barley diet was determined by means of the quinhydrone electrode. The urine of the rabbits on the alfalfa diet was too alkaline for the range of this electrode. The pH was determined colorimetrically with thymol blue. No degree of accuracy can be claimed for the alkaline urines, because of the heavy precipitate which they always contain. If the precipitate be removed by centrifugation, the pH of the supernatant liquid on dilution will not be the same as when the solids are present. After the first seven months of the experiment, the urines were examined for albumin and casts.

The plasma pH was determined on the plasma as drawn, as described in earlier publications (4). The total  $\text{CO}_2$  content was determined in the same sample.

## RESULTS

*Blood amino-acid and urea nitrogen.* Data for blood urea and amino-acid nitrogen were obtained for five of the rabbits on the acid diet just

before death. Both increases in the blood amino-acid nitrogen and liver changes as indicated by histologic examination were observed in two of the acid diet rabbits. The blood amino-acid nitrogens for the three other rabbits on the acid diet and those for three rabbits on the alkaline diet, determined at the same time as control values, were within the normal range. All the urea nitrogen values were within the normal range with the exception of that for the rabbit which had spontaneous focal nephritis. Data for this rabbit were obtained the day preceding and the day of death. It is interesting that the day preceding death both values are within normal limits, the very rapid rise (urea from 38.5 to 89.3, amino-acid nitrogen from 9.4 to 18.2) occurring in less than twenty-four hours. No significance can be attached to this isolated observation.

*Weights of liver and kidneys.* Since the rabbits on the barley diet died at intervals over a considerable period of time, there is no basis for comparison of the weights of the organs with the organs of rabbits on the alfalfa diet at the corresponding time. The weights were nevertheless recorded. The average for both kidneys for the rabbits on the barley diet was 14.1 gm., for the rabbits on the alfalfa diet, 20.2 gm. The average body weight for the former was 2.6 kilos., for the latter, 3.7. The size of the kidneys followed roughly the body weight. For the liver, the converse was true, the livers of the rabbits on the barley diet averaging 84.8 gm., those of the rabbits on the alfalfa diet, 72.0 gm.

*Urine pH.* The pH of a 24-hour urine specimen was determined each time the blood pH was taken. For the rabbits on the barley diet, the range of urine pH was 5.2 to 6.2. For the rabbits on the alfalfa diet, the range was 8.2 to 9.6. The individual values are not reported since they are of no significance. None of the urines of the alkaline series showed either albumin or casts at any time of the experiment. The urines of the acid series invariably gave slight positive tests for albumin. Casts were rarely found. The individual findings are not reported since there were no exceptions to the generalization.

*Plasma pH.* Of five rabbits on the acid barley diet<sup>1</sup> from 16 to 23 months,

<sup>1</sup> Another series of ten rabbits was given exclusively unpolished rice. The range of urinary acidity was pH 5.0 to 6.0. After five months, evidence of vitamin deficiency was marked, and after ten months six of the rabbits had died. Plasma pH and total CO<sub>2</sub> were determined five months after the rice diet was begun.

Plasma pH: 7.48, 7.41, 7.40, 7.42, 7.47, 7.45, 7.43. Mean  $7.44 \pm 0.01$ .

Plasma total CO<sub>2</sub>: 46.4, 39.0, 50.0, 36.9, 44.9, 46.1, 50.1. Mean  $44.8 \pm 2.2$ .

The values show no significant deviation from the initial control values. While the time interval for the rice series is much shorter than for the barley series, the inferences to be drawn from the experiments are the same; during a period of gross dietary abuse carried to the point of death, the acid base equilibrium of the blood remained unchanged on a diet high in acid ash.

only one showed evidence of a terminal acidosis. Histologically there was a focal spontaneous nephritis. Even including the data for this rabbit, the mean for the five terminal total plasma  $\text{CO}_2$  values for the acid diet rabbits is  $49.2 \pm 4.2$  volumes per cent, while that for the value for six rabbits at the beginning of the experiment was  $47.2 \pm 3.6$  volumes per cent. Likewise, the pH values, including the rabbit with the kidney damage, show no change, the mean for the terminal acid diet rabbits being  $7.44 \pm 0.05$ , that for six rabbits at the beginning of the experiment,  $7.45 \pm 0.02$ . In examining the total plasma  $\text{CO}_2$  and pH data for the rabbit, one notes a large normal variation for the same rabbit from day to day, with an occasional erratic result. It is possible that the occasional erratic result obtained was due to temporary injury to the heart by puncture. The final results, both chemical and histologic, indicated no permanent injury. An example is Rabbit 6, on the alfalfa diet. The total  $\text{CO}_2$  during two years fluctuated between 39 and 46 volumes per cent with one low value of 25.0 per cent. In human beings such a variation is not encountered, there apparently being a more delicately balanced mechanism for maintaining a narrow range. The normal plasma pH range for human venous blood determined by us with the quinhydrone electrode is 7.40 to 7.50. From data taken from fifteen different subjects, Cullen and Earle (5) obtained exactly the same range. Our values for the arterial blood of the rabbit cover the same range overlapping both extremes by 0.05 pH.

TABLE I

BLOOD UREA AND AMINO-ACID NITROGEN VALUES FOR BARLEY SERIES PRIOR TO DEATH WITH CONTROL VALUES FROM ALFALFA SERIES.

Rabbit	Date	Urea N.	Amino-acid N.
201, Barley	June 11, 1930	38.5	9.4
	June 12, 1930	89.3	18.2
203, Barley	June 11, 1930	19.6	—
210, Barley	Aug. 6, 1930	33.3	15.5
202, Barley	Jan. 7, 1931	12.9	10.2
212, Barley	Jan. 7, 1931	25.0	11.6
204, Alfalfa	June 12, 1930	19.8	9.4
205, Alfalfa	June 12, 1930	27.8	9.3
206, Alfalfa	June 12, 1930	30.3	9.7

The total plasma  $\text{CO}_2$  of the rabbits maintained on alfalfa was not significantly changed at the end of two years. The mean for five rabbits was  $41.9 \pm 2.5$  volumes per cent, which is lower by a little over once the standard deviation of the mean than the initial control value of  $47.2 \pm 3.6$  volumes per cent. Likewise, the plasma pH values show no change, the mean for the six terminal values being  $7.46 \pm 0.02$ .

TABLE II  
ACID BASE DATA AND HISTOLOGY OF RABBITS ON PROLONGED BARLEY OR ALFALFA DIET

Diet and Rabbit No.	pH and Total Plasma CO <sub>2</sub> of Blood											Histology			
	Feb. 1929	March* 1929	Apr. 1929	May 1929	Sept. 1929	Dec. 1929	March 1930	June 1930	Aug. 1930	Jan. 1931	March 1931	Kidney	Liver	Aorta	Heart Coronary
Barley 1	7.46	7.46 46.0	7.50 40.2	7.50 47.3	7.36 35.5	7.43 37.0	7.42 34.0	7.27 <sup>d</sup> 35.0				Spon. focal nephritis	Cirrhosis	—	—
2	7.48	7.48 50.6	7.54 46.7	7.44 46.4	7.49 47.9	7.48 45.6	7.44 33.6			7.44 <sup>b</sup> 56.7		—	Slight fatty change	—	—
3	7.51											—	—	—	—
4		7.43 49.4	7.49 52.6	7.53 57.7	7.45 47.0	7.45 41.8	7.48 40.1	7.58 <sup>k</sup> 50.1				—	—	—	—
10									7.48 <sup>b</sup> 58.7			—	Moderate fatty change	—	—
12										7.44 <sup>k</sup> 45.8		—	—	—	—
11							<sup>d</sup>								

\* Average of 4 determinations made weekly.

<sup>d</sup>—Died.

<sup>x</sup>—Killed.

TABLE II (Continued).

Diet and Rabbit No.	pH and Total Plasma CO <sub>2</sub> of Blood											Histology			
	Feb. 1929	March* 1929	Apr. 1929	May 1929	Sept. 1929	Dec. 1929	March 1930	June 1930	Aug. 1930	Jan. 1931	March 1931	Kidney	Liver	Aorta	Heart Coronary
Alfalfa 4	7.36 32.8	7.44 43.9	7.48 43.7		7.52 49.8		7.45 48.4	7.48 43.7			7.45κ 39.7	—	—	—	—
5	7.43 49.7	7.42 41.5		7.44 46.7	7.45 40.4	7.47 41.7	7.47 43.6	7.45 39.5			7.45κ 39.5	—	—	—	—
6	7.44 46.2	7.44 41.6	7.40 25.2	7.42 40.3	7.47 40.2	7.41 40.4	7.46 41.5	7.47 44.2			7.47κ 39.0	—	—	—	—
3											7.49κ 51.8	—	—	—	—
9									7.39κ			—	—	—	—
30									7.52κ 39.6			—	—	—	—
7										D		—	—	—	—

\* Average of 4 determinations made weekly.

D—Died. κ—Killed.



### *Histology*

Autopsies were made within a few hours after death and tissues from the kidney, liver, aorta and heart were fixed in Zenker's solution, embedded in paraffin, and stained with hematoxylin and eosin. The results of the histologic examinations are briefly recorded in Table II, the minus sign indicating that no change is noted. In sections of the kidneys of Rabbit 1, on the barley diet, there is the usual picture of a slight degree of spontaneous focal nephritis. There are dilated tubules with flattened or desquamated lining cells, focal regions of small, round cell infiltration in which the tubules are compressed and atrophic, and unchanged glomeruli and blood vessels. This condition has been carefully described by Bell and Hartzell (6), by Leiter (7), by Le Count and Jackson (8), and others.

In the livers of Rabbits 2 and 10 there are slight and moderate degrees of fatty change, respectively. In the higher grade of involvement, apparently not more than 20 per cent of the hepatic parenchyma is affected. The spontaneous occurrence of the liver changes described is sufficient to account for their presence, and so no causal relationship can be attributed to the diet.

### DISCUSSION

The results of the present study show that the rabbit is not as sensitive to changes in acid ash or alkaline ash intake as has been supposed. On the alkaline side he is better able to cope with alkali excess than is the human being, a constant passage of a urine in the range pH 8.2 to 9.5 having no effect upon the plasma pH or CO<sub>2</sub> content. The range of urine acidity on the barley diet, pH 5.2-6.2, is one which produces no shift in the acid base equilibria in the blood of humans. Whether the rabbit is more sensitive to excess acid than the human can not be decided from these studies. Since rabbits on a prolonged liver or oats diet develop degenerative blood vessel changes, while those on a barley diet do not, the range of urinary acidity for all three diets being the same, one is forced to ascribe the changes produced by the liver and oats diet to some factor other than the acid ash. If the barley diet, which was obviously deficient, had produced such changes, the proof would still be lacking that the effects were not secondary, due to some such factor as vitamin deficiency or infection. The experiments with barley show rather dramatically that in spite of gross dietary abuse carried to the point of death, the acid base equilibrium remained normal to the end.

### SUMMARY

There was no significant change in the plasma pH or CO<sub>2</sub> content of the blood of rabbits maintained on a barley diet for a period of nearly

two years, as compared with rabbits on an alfalfa diet, the urinary acidity of the barley group ranging from pH 5.2 to 6.2, that of the alfalfa group from pH 8.2 to 9.5.

No pathological changes were noted for the seven rabbits maintained on alfalfa. Early death (16 to 23 months survival) with clear cut evidence of diet deficiency was noted for the barley diet, fatty change of the liver being present in three of six rabbits and a focal diffuse nephritis in one.

We are indebted to Miss Elsie Hill for the CO<sub>2</sub> analyses and to Doctor Albert H. Elliot for some of the histologic examinations.

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## ACTION OF RADIO-ACTIVE SUBSTANCES ON VITAMINS\*

By

ALBERT G. HOGAN, CHARLES L. SHREWSBURY, GERALD F.  
BRECKENRIDGE, AND WALTER S. RITCHIE

*(From the Departments of Animal Husbandry, Agricultural  
Chemistry, and General Chemistry, University of  
Missouri, Columbia)*

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THE Department of Chemistry frequently has in its possession preparations of radio-active materials, so it seems desirable to take the opportunity of studying their action on vitamins. A number of workers have studied the action of rays of short wave length on these substances, and it is clear that at least one of them, vitamin A, is quite labile.

Zilva (1) exposed milk fat to the rays from a quartz mercury arc, and observed that it was bleached, acquired the odor of tallow, and became unpalatable. Vitamin A was destroyed. In a later study (2) he concluded the destruction of the vitamin was due to ozone, for if the exposure were conducted in an atmosphere of carbon dioxide there was no loss of vitamin activity. Spinka (3) subjected milk to a similar procedure, and concluded that toxic substances developed which killed the experimental animals, but vitamin A was not destroyed. Zilva (4) remained convinced, however, that in his experiments destruction of the vitamin had occurred. Titus, Hughes, Hinshaw, and Fitch (5) exposed milk to the rays of a quartz-mercury arc, and observed destruction of vitamin A. Supplee and Dow (6) irradiated milk for very short periods, a few seconds up to 3 ½ minutes, but were not able to detect any destruction of the vitamin. Steenbock and Coward (7) regarded vitamin A as remarkably stable to ultra-violet rays. The effect of rays of still greater frequency has apparently received little attention.

As to the effect of rays of short wave length on the water-soluble vitamins there is at present no unanimity of opinion. Hogan and Hunter (8) reviewed the earlier literature on this topic, and reported that one of the factors in the vitamin B complex, stable to heat, is destroyed by ultra-violet irradiation. No attempt was made to determine whether the destruction was complete, or whether partial destruction of other factors occurred. Chick and Roscoe (9) confirmed this report in its essentials, and

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agreed that one factor, G ( $B_2$  in their terminology) undergoes very considerable destruction. In addition they observed that B( $B_1$ ) is also partly destroyed. An attempt (10) was made in another department of this institution to apply the technic of Hogan and Hunter to a study of the distribution of vitamins B and G in certain foodstuffs. The behavior of both control and experimental animals indicated that the method could be used successfully, but after several months the observations were interrupted. On resuming these studies, all attempts to repeat the previous work apparently met complete failure. The only alteration in the experimental conditions mentioned was a change in the yeast supply. Palmer and Kennedy (11) were unable to detect any destruction of either factor. Guerrant and Salmon (12) estimated from their experience that approximately 25 per cent of vitamin G is destroyed by irradiation, and evidently additional study will be required to explain the discrepancies. Hottinger (13) reported that the vitamin C content of whole dried milk was not reduced by ultra-violet radiation.

It was established by Steenbock (14) and Hess (15) some time ago that foodstuffs acquire antirachitic potency on irradiation with ultra-violet rays. There is still some dispute as to the possible precursors of vitamin D, but the concensus of opinion supports the view of Rosenheim and Webster (16) that ergosterol is the parent substance. According to Knudson (17) ergosterol may also be activated by cathode rays.

Morrison, Peacock, and Wright (18) state that x-rays have a destructive effect on vitamin D.

#### EXPERIMENTAL

Both radiothorium and mesothorium were available at various times, and they were used interchangeably, depending on which was available at the time. They were in the form of chlorides or bromides and, though highly concentrated, they were far from being chemically pure. The radioactive materials were loosely cemented to the inside of small containers, either crucibles or flat crystallizing dishes, and covered with mica to prevent loss of the active substance. This prevented bombardment by alpha particles, and reduced in some degree the intensity of the beta-rays. The activity of the various preparations, measured by the gamma-ray method, varied between 20 and 25 milligrams of radium.

Albino rats, reared on Steenbock's (19) ration, were used as experimental animals. In some cases these were transferred to the vitamin-A-free diet at 19 days of age, a procedure suggested by Nelson (20). In our experience this modification greatly reduces the variability among test

animals. The length of the depletion periods becomes more uniform, and practically all individuals develop xerophthalmia within a few days of each other. The composition of one of our experimental diets, No. 925, is given below.

	Per Cent
Egg white	20
Dextrin	64
Dried yeast	9
Mineral salts	4
Agar	2
Irradiated cotton seed oil	1

The composition of No. 924 was the same, except that yeast was eliminated and the dextrin increased to 73 per cent. Yeast was supplied separately, 500 mgm. daily.

Our first observations were made on vitamin A, and butter was the chief source of material. It was placed in a low crystallizing dish, 9 cm. in diameter, in a layer about 0.8 cm. deep. The radio-active substance was placed directly over this, with the mica about 2.5 cm. above the butter, and allowed to stand for 96 hours. After 72 hours a thin area immediately beneath the container was distinctly bleached, and 24 hours later it was practically colorless. The butter was then transferred to a closed glass container and stored in the refrigerator. The bleaching process continued, however, for when examined later the entire sample was colorless.

The rats used for the vitamin tests were approximately 23 days old when selected and weighed about 40 grams. They were placed on Ration 925, free from vitamin A, for a depletion period, usually about 5 weeks. The experimental period was begun when the animals began to lose weight, though usually some ophthalmias had also appeared. The animals were then divided into three groups, the first of which was continued on Ration 925. The purpose of this group was to determine the survival period of rats on a ration that is practically free of vitamin A. The other animals were changed to Ration 924, deficient in both vitamins A and B. It was anticipated that the exposed butter might be unpalatable, and so would not be consumed readily. It was hoped that if the vitamin B supplement, dried yeast, were mixed with the butter, complete consumption would be secured. This expedient may have been unnecessary, but at any rate there were no difficulties because of failure to consume the supplements. These were weighed out daily into small glass containers. Group II received treated butter, and Group III received the untreated material. Both

samples came from the same package, and were stored under the same conditions. Each rat in a group had a litter mate in the others, of the same sex and of approximately the same weight.

It is evident from Fig. 1 that the exposed butter gave no evidence of vitamin A activity, as judged by rate of growth, incidence of ophthalmia, or survival period. The behavior of the animals was practically identical with that of the negative controls. The positive controls were either cured of ophthalmia, or protected.

Cod liver oil was also exposed to radiothorium, and though no change in the oil was visible, it apparently lost its vitamin A potency. Beginning on the 60th day, Rat 2824 (Fig. 1) was given a daily dose of 14 mgm. of the exposed oil, without affecting in the least the course of the disease. During the last 8 days shown on the graph, Rat 2833 was given similar treatment, with the same result. Numerous trials, not recorded here, have shown that this amount of the untreated cod liver oil is more than sufficient to meet the vitamin A requirement of the rat.

As previously mentioned, the bleaching process in butter continued after removal from the vicinity of the radio-active substance. This suggested that the destruction of the vitamin may not be due directly to exposure to the radio-active material, but is an indirect effect. This may be explained as due to an acceleration of the changes that normally occur, or to the formation of some unusual substance which has a destructive action. It was decided, therefore, to mix an exposed, non-potent sample of butter with an equal weight of untreated material, and test the potency of the mixture after standing for various periods of time. A portion of the untreated butter was also set aside for comparison.

The procedure used in making the tests was varied a little from that previously described. At the end of the depletion period, all rats were transferred to Ration 924. Each received the same amount of dried yeast, and of the fat supplement. For Group I this was 200 mgm. of hydrogenated vegetable oil.<sup>1</sup> Group II received 200 mgm. of a mixture of equal parts of exposed and untreated butter. Group III received a mixture of equal parts untreated butter and the vegetable oil. In the earlier trials no diminution of vitamin A activity was detected, and these rats are not shown in Fig. 1. In the first trial the mixture was prepared the same day the test feeding was begun, and continued for 28 days, without obtaining any evidence of vitamin A insufficiency. In the meantime the negative controls had developed severe ophthalmia and died. All butter supplements were then

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<sup>1</sup> Crisco.

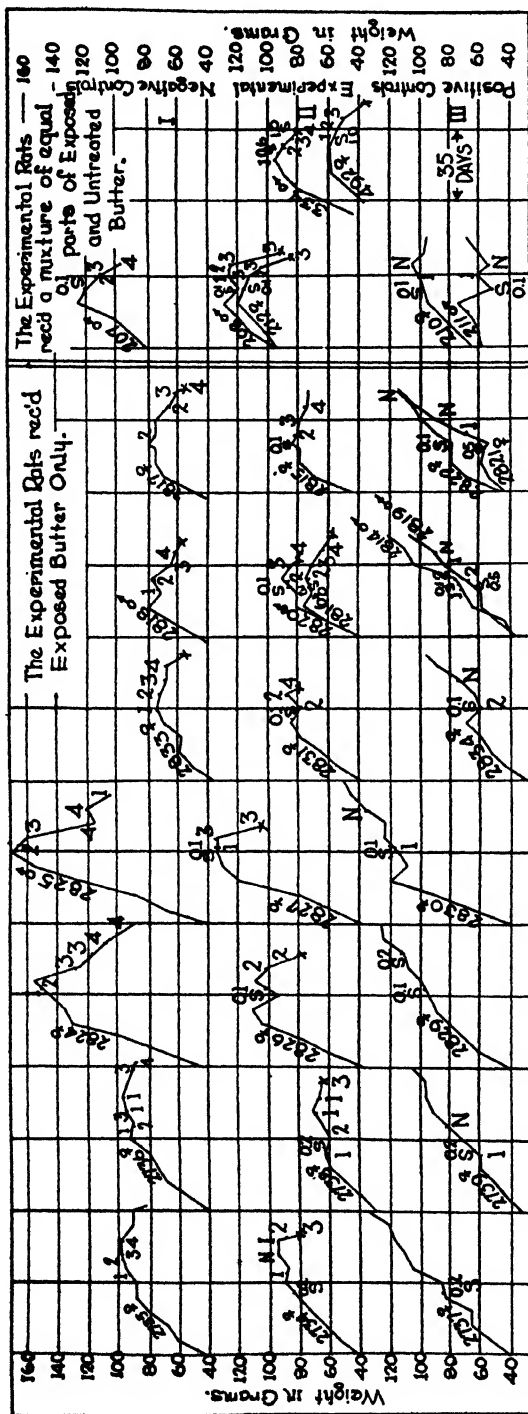


FIG. 1. Rats in the upper row received no vitamin A supplement. All experimental rats received butter that had been exposed to radio-active substances. Of these animals, Nos. 208, 212, 334, and 492 received a mixture which contained, in addition to the treated material, an equal amount of untreated butter. The mixture had been stored 200 days when first used. The animals in the lower row received untreated butter.

The point at which the supplements were first fed is indicated by the letter S. The amount of the supplement is indicated in grams just above, or below the letter. The severity of the ophthalmias is indicated by the numerals, 1, 2, 3, and 4. If an animal recovered from ophthalmia the fact is indicated by the letter N. Deaths are indicated by the mark, x.



discontinued, and the remaining 5 rats were each given a daily dose of 14 mgm. of the treated cod liver oil. By the thirteenth day following, one of them had developed a marked ophthalmia, and was losing weight. It was then given the untreated oil, and 7 days later had entirely recovered. By this time the other 4 rats had developed ophthalmias and were also losing weight.

In the second trial the mixture was prepared April 22, 1929, and the test feeding began August 2. The mixture was almost completely bleached within 8 weeks, and was apparently colorless when the experiment began. Again no destruction of vitamin A potency was detected, though slight loss may have occurred. In a subsequent trial this same material, mixed on April 22, was used after standing for 200 days. By this time the vitamin activity of the sample had entirely disappeared. Rat 334 received 0.6 grams daily, and Rat 492 received 1.0 gram, but neither ophthalmias nor survival periods were apparently affected. This is a relatively slow rate of destruction, and it seems unlikely that the disappearance of the vitamin is the only change that occurs. One may assume that the vitamin is destroyed by intermediate compounds that are formed in the fat.

Another observation worthy of comment is the disappearance of the vitamin A color test as given by the antimony trichloride reagent (21). Untreated cod liver oil gives a brilliant reaction, but at the time the biological examinations were made the exposed preparation gave a negative test<sup>2</sup> with the color reagent.

It is evident that destruction of the vitamin in butter proceeds much more rapidly while exposed to the radio-active material, but our data do not show how much time is required for complete destruction. The exposures were made some weeks before the animals were ready for the test feeding, and we are unable to say whether or not the losses in color and in vitamin A potency take place at the same rate. We will also reserve, until a later date, any expression as to the effect of radio-active substances on cod liver oil. When the biological test was made the oil had become quite viscous and it was noted that the experimental sample occupied a considerably lower percentage of the volume of the container than did the control. The smaller sample was therefore exposed to a relatively larger volume of air, and both the increase in viscosity and the destruction of vitamin A may have been due merely to contact with oxygen.

As to which of the rays is the active agent, it seems probable this is a property of the beta-ray. All preparations were protected behind mica,

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<sup>2</sup> These observations were made by Dr. J. E. Hunter.

which shields out the alpha-particles. Milk fat was later covered with a lead plate, then exposed to mesothorium. This would shield out all but a small part, possibly 5 per cent, of the entire beta-ray activity, but would reduce the gamma-ray activity scarcely at all. The milk fat exposed in this manner was not perceptibly bleached, and no decrease of vitamin A activity was detected. It is worthy of note that Hanson and Heys (22) found that the lethal mutations produced by exposing *Drosophila* to the rays of radium are due entirely to the beta and not at all to gamma rays. It is possible, however, that the two phenomena, bleaching, and loss of vitamin A potency, are more or less independent.

In addition, observations were also made to determine whether vitamins B, C, D, and G are labile when exposed to radio-active substances. The Osborne-Wakeman (23) concentrate was used as a source of both B and G. Concentrated orange juice<sup>3</sup> was used as a source of vitamin C. Both cod liver oil, and a commercial preparation of activated ergosterol,<sup>4</sup> were used as sources of vitamin D. No destruction was detected in any case. Ergosterol was readily activated by ultra-violet rays so an effort was made to activate it by exposure, under similar conditions, to mesothorium. Some of the exposures were for 6 hours, some for 96, but no antirachitic potency could be detected. In as much as the results were negative, the procedures are not described in detail.

### SUMMARY

1. Vitamin A of milk fat is destroyed by exposure to radio-active substances. The destruction may be indirect.
2. The beta-ray is apparently the active agent.
3. The evidence indicates that vitamins B, C, D, and G are stable when exposed to beta or gamma rays.

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<sup>3</sup> Supplied by the California Orange Grower's Association.

<sup>4</sup> Acterol. We wish to express our appreciation of the kindness of Dr. C. E. Bills of Mead Johnson and Co., who supplied the acterol and ergosterol, and gave valuable advice as to methods of activating ergosterol with ultra-violet rays.

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## SALINE AND ALKALINE DRINKING WATERS

BY VICTOR G. HELLER

*(From the Department of Agricultural Chemistry Research, Oklahoma Agricultural and Mechanical College, Stillwater)*

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A NUMBER of years ago a preliminary report (1) was made of the effect of saline drinking waters upon growth and reproduction of animals. Since that time the problem has been continued and has increased in interest as well as in scope and practical application. As stated in the previous article, of the several nutriments needed by man and animals, drinking water is undoubtedly the greatest variable in this day of rapid travel and the one most difficult of correction in many regions of the United States. There are large areas of the earth's surface where deep wells cannot be used due to the extreme alkalinity or salinity of waters procured. Many streams flow through salt beds on the surface or through areas where they become contaminated with the waters from oil wells, factories or mines. When the flow of water is limited, due to dry seasons, the dissolved salts make the use of these waters questionable. In other sections alkali beds of great area become leached by rains or underground waters, causing the water to be even more deleterious for birds or aquatic life.

A search of the literature, as previously stated, reveals little to answer the question of a possible standard for drinking purposes and the physiological effects of an excess of salts. The U. S. Public Health Service (2) has set up an arbitrary standard for interstate carriers, which may be an ideal to be sought but one not possible to obtain in many regions. The pharmacologic literature is very complete in regard to the effect of minimum lethal doses of specific salts but is of little use in postulating the effect of a continued use of these salts in drinking waters. Volumes have been written by developers of mineral springs lauding their merits, especially in the literature of a generation ago; but as stated by Van Noorden (3), "The truth is that so little is known of the bearing of mineral waters on biological processes that most of the statements in biological literature may be stigmatized as idle make-believe." There are many articles having some bearing on the problem, but they are of no direct importance in the solution of our difficulty. In the medical literature we find references to the effect of certain waters in the stimulating of intestinal secretions, their effect upon digestion, and upon hydrogen ion concentration of the body fluids. Chase (4) makes a report of the apparent health of people living in

hard water sections of Ohio compared to the health of those living in soft water regions. Roberts (5) states that young fish can stand Cl up to 5,750 parts per million; while Young (6) believes that a shift in the osmotic effect to about six atmospheres, due to total salt, is fatal. Shaw (7) in his duck disease studies of salt areas makes record of the amount of soluble salts found necessary to injure and kill migrating ducks.

A few articles having more direct bearing upon the subject have appeared in the literature since the publication of our first article. These pertain to deaths thought to have been caused by saline waters in the British Colonies. Legg (8) describes the death of sheep that accidentally drank salt solutions while being shipped. Ramsay (9) states that cattle in cases can live on water containing 17,120 p.p.m. of total salt and thrive on 11,400 p.p.m. Horses thrive on waters containing up to 5,720 p.p.m. and can be sustained on 9,140 p.p.m., if not worked too hard; while sheep are similar to cattle in this respect. Scott (10) gives a rather detailed description of the clinical symptoms of cattle suffering from the effects of salty waters.

Clough (11) and Mitchell, Carl and Carman (12) refer to deaths of poultry from the excessive consumption of ordinary table salt. We have observed in many cases deaths among hogs and cattle that had been overfed fine salt while in a salt-hungry condition. Hogs, especially, seem to be particularly susceptible. In most of these cases, however, we are dealing with the effect of single large doses of salts in salt solution or with unknown mixtures. In our problem it is necessary to know the effect of continuous use of such solutions over long periods of time. Likewise, it is necessary to know whether there is possibly some physiological change whereby the body can adjust itself to these altered conditions. Such a change has been noted (13) in freezing points of blood of eels after immersing them in saline water, indicating that some salts are evidently absorbed and retained in the blood. The writer has often found both man and animal sustaining no apparent injury from continuously using waters that caused very serious discomfort and in some cases death to animals not accustomed to such concentrations.

The purpose of this investigation has been to demonstrate what concentrations of various commonly occurring salts and alkalies produced death, stunted growth, inhibited reproduction and disturbed lactation; to determine by mixture of salts if there is an antagonistic reaction between salt ions as has been proved to be effective for plants; to ascertain if there is some physiological readjustment by which animals can become accustomed to saline drinking waters; and lastly, to make a study of the dele-

terious condition produced and mechanisms by which physiological conditions are altered.

#### *Method of Procedure*

In all related studies undertaken by other workers, it has been the custom when deaths occurred to obtain a sample of the water which produced the effect and to make an analysis of it. In these cases it was then necessary to postulate what was the effect of the mixture. It has seemed advisable in our case to proceed by a method previously used in plant nutrition studies, that is, to use distilled water and add varying amounts of the salt under consideration until the deleterious levels were determined. Rats were used as experimental animals for all preliminary tests, because of convenience of control, the possibility of using very large numbers, and comparatively short reproductive periods. The animals were housed in cylindrical wire cages commonly used for nutrition tests, and fed a well-balanced ration (consisting of yellow corn, whole wheat, milk powder, alfalfa, cottonseed meal, and tankage) regularly used for our breeding colony. One per cent of sodium chloride and 1 per cent of calcium carbonate were added for mineral supplements. The drinking water was furnished from glass bottles fitted with glass syphons. The usual precautions in regard to measuring the amount of water and feed consumed, the recording of weights of all animals, and the practice of sanitary measures familiar to nutrition workers were observed.

The first studies were made with sodium chloride, that being the most widely distributed salt in most regions. Ten solutions containing sodium chloride were prepared as follows: 0.5, 1.0, 1.25, 1.5, 1.75, 2.0, 2.25, 2.5, 3.0, and 4.0 per cent. Ten cages containing 4 or more rats each, previously selected so as to be as nearly comparable as possible, were started and given the various solutions as a sole source of drinking water. Within a few days deaths occurred among the higher levels, and within 10 days no rats receiving 2.5, or more per cent salt were alive. It was interesting to note that at lower levels the amount of water consumed became greater with an increasing amount of salt until a concentration was reached which they refused to drink at all, refraining from drinking until thirst finally compelled them to drink a large quantity at one time causing death in a short time. Farmers often give a similar description of cattle dying apparently from salty waters. It was observed that waters containing 1.5 per cent were often fatal to young rats. Growth stopped, the animals presented an emaciated appearance, the coats became rough, eyes were often sore, presenting a condition resembling ophthalmia, and diarrhea was always

present. An autopsy showed the intestines to be inflamed and, in severe cases, bloody. In many cases, spots were found on liver and lungs. The sites of infection, we believe, are due to a secondary cause brought about by the general weakened condition. It was later demonstrated that when the animals on levels up to 1.5 per cent salt survived the preliminary stunted period, they resumed growth and in many cases reproduced and reared their young in a manner somewhat comparable to normal rats. A few survived at higher levels, although the ability to nourish young was very limited. Occasionally, normal young were born, but they often died seemingly due to a lack of action of the mammary glands. Therefore, it is quite apparent that there is some physiological readjustment which should explain how man and animals reared in certain sections thrive on waters that kill or injure those accustomed to the drinking of soft waters only. It was also observed in the course of the investigation that old animals were more resistant to these changes than young. Rats four weeks of age and weighing 50 grams, when placed on 1.5 per cent salt nearly always died. The ones surviving passed through a stunted period, in time started growing, and in some cases grew normally. Full grown animals lost weight but rarely died. We believe this explains why a herd of cattle shipped into these regions will die or be seriously injured, while other animals accustomed to these conditions will live in apparent good health. Likewise, aquatic fowls will rear young on saline lakes, while migrating birds from clear water regions will die by the hundreds in such lakes. The writer's attention has been called to fish living in salt water wastes of oil wells, this water containing over 1 per cent of sodium chloride alone as well as other salts. Yet, fish from surrounding streams would have died if placed in such concentrations.

In working with plant nutritive solutions, it has been found that at a certain level sodium chloride becomes toxic. To this concentration calcium salts could be added, and despite the increased concentration the plant could live. This is explained on the basis of antagonistic action of ions. In order to determine whether this would be true for animals, other salts were added to the dangerous levels of sodium chloride. The additional salt made the previous toxic salt more toxic. Evidently, either antagonistic ion action is not effective for animals, or some other factor predominates in the final results. Animals are also different in that they can use much greater concentrations of salts than plants. All of our observations lead us to believe that there is an osmotic rather than a toxic reaction of salts. The accompanying table shows the quantities of the various salts used, the number of test animals, the effect upon reproduction, the number of

TABLE I  
RESULTS OBTAINED BY USE OF SALINE WATERS AS A SOLE SOURCE OF DRINKING SUPPLY FOR RATS

Salt	Per cent	Males	Females	Litters	Young born	Young lived	Young died	Remarks
NaCl	0.5	2	3	3	24	20	4	Growth normal.
	1.0	1	1	1	7	4	3	Growth normal.
	1.5	3	5	5	39	31	8	Growth subnormal. Some die.
	2.0	2	3	3	20	17	3	Young die.
	2.2	1	2	0	0	0	0	Old and young die.
	2.5	4	6	0	0	0	0	Old and young die.
	3.0	2	2	0	0	0	0	Sudden loss in wt., diarrhea, rough hair, and death.
	3.5	2	2	0	0	0	0	Young and mature die soon.
	4.5	4	2	0	0	0	0	Die at once.
NaCl+KCl	1.5							
	0.7	2	3	3	39	0	39	Young die within 15 days.
	1.7							
	0.6	2	2	0	0	0	0	Old ones die.
	2.5							
	1.0	2	4	0	0	0	0	Old and young die.
	3.0							
NaCl+CaCl <sub>2</sub>	1.5							
	0.7	5	4	1	7	0	7	Hair thin and rough. No gain.
	3.0							
CaCl <sub>2</sub>	1.5	4	4	0	0	0	0	All die at once.
	1.0	2	2	2	13	10	3	General condition good.
	1.5	3	6	7	77	62	15	Growth satisfactory. Condition good.
	2.0	1	2	5	30	6	24	Interferes with lactation.
MgSO <sub>4</sub>	2.5	1	1	0	0	0	0	Old ones die.
	0.5	2	2	2	10	10	0	Growth and reproduction.
	1.0	1	3	6	51	24	27	Young poor; old emaciated.
	1.5	3	3	3	20	13	7	Growth subnormal; diarrhea, rough coat.
MgCl <sub>2</sub>	1.0	2	4	2	15	1	14	Condition of old not normal.
	1.5	1	2	0	0	0	0	Growth below normal.
MgCl <sub>2</sub> +CaCl <sub>2</sub>	1.0							
	0.5	2	2	1	7	0	7	Growth of old normal. Lactation poor.



TABLE I (Continued)

Salt	Per cent	Males	Females	Litters	Young born	Young lived	Young died	Remarks
MgAc	1.5	2	2	2	17	8	9	Growth satisfactory. Reproduction.
Na <sub>2</sub> CO <sub>3</sub>	1.0	3	7	7	45	31	24	Growth of offspring unsatisfactory.
	1.5	1	7	2	19	0	19	Young die; old rough coated, red eyes, diarrhea.
NaHCO <sub>3</sub>	1.5	2	4	7	55	48	7	Old somewhat undersize. Growth of offspring impeded.
	2.0	1	2	2	14	7	7	Growth and appearance unsatisfactory.
NaOH	0.5	2	4	0	0	0	0	Practically normal growth.
	1.0	3	4	0	0	0	0	Little growth, animals dirty, very nervous, diarrhea, sore eyes.
Ca(OH) <sub>2</sub>	sat.	4	4	4	30	19	11	Some interference with growth during lactation. 3 generations produce.
CaSO <sub>4</sub>	sat.	3	8	7	33	28	5	Satisfactory growth.
Na <sub>2</sub> SO <sub>4</sub>	1.5	1	2	1	11	6	5	Appearance of adults satisfactory.
NaF	1.0	1	2	0	0	0	0	Growth poor. Animals emaciated.
KI	1.0	1	2	0	0	0	0	Growth fair. No reproduction.

young reared, and the general condition of test animals. It also illustrates the results of introducing a mixture of salts into drinking waters. It should be noted at this point that these results have been checked and rechecked over a period of four years. Whenever reproduction occurred, the young were continued on their mother's diet through five or six generations.

The effect of seasonal variation was also given consideration. As one might predict, the hot dry days of summer seemed to be most fatal to animals drinking saline waters, a result undoubtedly due to a greater thirst and therefore greater salt intake.

In streams contaminated by oil well wastes, calcium chloride and

magnesium chloride are often found in large quantities. Examination of these wastes entering streams often shows a salt content of over 20.0 per cent. In many sections near the gypsum beds, waters saturated with calcium sulfate are common; while sodium carbonate and bicarbonate frequently occur in several western states. For these reasons these salts were studied separately and in combinations, as is shown in the table. Calcium chloride is not normally found in most waters, but it is one of the common salts in oil well wastes. Streams and pools in the neighborhood of oil fields are often heavily saturated with this salt. A series of experiments as conducted with sodium chloride were carried out, using calcium chloride as dissolved salt. It was expected that more deleterious effects would be observed, but the contrary was established. An inspection of the table will reveal that at 1.5 per cent, rats, after the first rather difficult adjustment period, grew normally, reproduced their young, and were able to suckle them through the lactating period. In fact, the general appearance, coat, eyes, and build of the rats seemed superior to those of the breeding colony. All of our tests indicated that calcium chloride might prove to be a more satisfactory source of calcium in mineral mixtures than calcium carbonate. Even at 2.0 per cent, growth after the introductory period was normal, but lactation was hindered. At higher levels, results similar to those with sodium chloride were encountered.

Waters saturated with  $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$  are common about gypsum deposits. However, this salt is only soluble to the extent of 0.24 per cent. Waters of this type have not proved to interfere with normal growth, and reproduction.

Magnesium, the third ion quite commonly found in salt well wastes in the form of magnesium chloride, and in many well waters near sulfate deposits as magnesium sulfate, was used in the form of magnesium chloride, sulfate, and acetate. An examination of the tables will demonstrate that magnesium is not so satisfactorily used as either sodium or calcium, but the true condition can only be appreciated by observation of the living animals. When the animals were first given these solutions there was a rapid loss of weight and severe diarrhea; and even in cases where the animals recovered, they did not have smooth coats and a general emaciated condition was presented. It is evident that magnesium in company with the sulfate radical is more detrimental than magnesium chloride and much more so than the acetate.

In many sections of the west, alkali waters containing  $\text{Na}_2\text{CO}_3$ ,  $\text{NaHCO}_3$ , and even  $\text{NaOH}$  are to be found. We have had occasion to analyze samples which contain large quantities of  $\text{Ca(OH)}_2$ . The latter is only soluble to

the extent of 0.18 per cent, but it was rather surprising to find that this hydroxide could be used as a sole source of drinking water through four generations without any evident deleterious results with the exception of a somewhat greater mortality of the young. Sodium hydroxide to the extent of 0.5 per cent permitted life and growth, but 1.0 per cent allowed no growth; the animals became thin and very highly nervous and irritable, the eyes became sore and diarrhea followed. A similar condition was likewise produced by 1.5 per cent of  $\text{Na}_2\text{CO}_3$ ; 1.0 per cent of the latter did permit growth and reproduction, but there was a high mortality of young. Sodium hydrogen carbonate, 1.5 per cent, permitted growth, reproduction, and rearing of young. These young, as well as the adult rats, were somewhat undersized. At 2.0 per cent the general appearance became unsatisfactory. With the sodium hydroxide and carbonate we found indications of factors other than osmotic pressure producing their effect; however, it was rather surprising to note the amount of alkali that could be utilized. A number of other salts have been used to answer certain specific cases called to our attention, but as they are not of general interest no comment need be added to the facts presented in the table.

The question has been raised whether or not data obtained from rats would be applicable to other animals. We have made sufficient isolated tests to furnish evidence that they are, at least for cattle, sheep, and rabbits. Other tests now under way will determine this conclusively and should it be proved that larger animals are exceptions to the rule, that fact will be reported at a later date.

Several series of very definite chemical analyses and measurements are also being run to answer definitely certain questions postulated in this article, namely, in regard to the change in osmotic effect, changes in pH of blood stream, and the effect on the organ size. A detailed description of the methods used and results will be forthcoming.

### CONCLUSIONS

1. Waters containing large amounts of salts in solution are deleterious for drinking purposes.
2. The effect produced seems to be more osmotic in reaction than due to any specific ion.
3. From 1.5 to 1.7 per cent seems to be the maximum amount of soluble salts that can be used with safety.
4. Animals seem much less susceptible to salt solutions than plants, and antagonistic effect of ions is lacking or is a secondary factor.

5. Chloride salts are less injurious than sulfates, and organic salts are less injurious than inorganic.

6. Alkalies are more deleterious than normal salts; evidently the osmotic effect is coupled to a harmful effect of the changed pH.

7. An interference in lactation and reproduction is noticeable even before the level producing stunted growth or death is reached.

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## Editorial Review\*

### THE INFLUENCE OF DIET ON RENAL AND BLOOD VESSEL CHANGES

COMPARATIVELY little of a positive nature is known concerning the effects of diet upon blood vessel and kidney changes, and in general diet is given secondary consideration or is discounted as a factor in the etiology of renal and cardiovascular diseases. Diet experimentation with laboratory animals has failed to supply the keynote to the solution of the clinical problems since species difference introduces a factor of unknown dimensions, but it has produced results of a nature to leave even the most skeptical uncomfortably in doubt. The wide scope of the subject, ranging from the more chemical aspects of nutrition to the highly specialized field of histopathology, adds to the difficulty of investigation. A study which is admirably presented from a histologic standpoint may leave much to be desired from a nutritional point of view. Certain important contributions arising from vitamin studies confine the pathological observations to descriptions of the gross. In considering evidence as conflicting as that associated with the problem under discussion, the reviewer has two alternatives: He may confine his efforts to cataloguing the evidence, a safe procedure but one producing a disjointed product, or he may attempt to evaluate and compare the work, often seeking for common conditions in apparently unrelated environments and for common causal factors in experiments involving uncontrolled variables. Before attempting the present review, tables of experimental data recorded in the literature were prepared, including such headings as number, age, and kind of animals, diet period, diet constituents, blood and urine chemistry, histopathology of kidneys, blood vessels, etc. The panorama of isolated facts and uncontrolled variables so produced struck home very forcibly the dangers encountered in correlation and the unwarranted deductions presented on many occasions.

Cholesterol, protein, acid ash, and vitamins (overdosage as well as deficiency) have been assigned a rôle in the production of kidney and blood vessel changes. For the sake of presentation, these four diet constituents will be considered separately, although it is obviously impossible to consider any one factor without including the others. The renal and arterial changes attending disturbances in purine metabolism (gout), which theoretically might be influenced by diet, can not be discussed because of

\* The author, Dr. Fritz Bischoff, is Chief Chemist and Chairman of the Research Committee, Santa Barbara Cottage Hospital, Santa Barbara, California.

the lack of experimentation and the sparsity of clinical observation. Tartrate nephritis and the sclerosis assigned to alcoholism, while properly included in the subject under discussion, will not be considered, as they represent isolated cases. Furthermore, no attempt will be made to discuss the extensive literature on the spontaneous occurrence of kidney and blood vessel changes in laboratory animals, studies which are obviously fundamental to an understanding of the changes produced by experimentation. Judgment should rest on statistics based on the incidence of renal changes in control and experimental groups and not on opinion as to whether changes observed at the termination of an experiment might be accounted for by spontaneous development. Unfortunately, some useless discussion has arisen concerning the supposed obstacle of spontaneous change which is more or less inherent in all biological research. In the present instance the etiology of spontaneous changes is not known. Infection is the most likely factor. In some of the early diet experiments aimed to produce kidney and blood vessel changes there was an obvious deficiency of vitamins, which led to a lowered resistance to bacterial invasion. The kidney and blood vessel diseases produced in experiments of this type were in some cases suspiciously like the spontaneous type of changes. For this reason especially, attempts to produce renal and blood vessel changes by diet must carry the assurance of adequate vitamin control before they may be seriously considered.

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Since vitamin A deficiency frequently causes changes in epithelial tissue, lesions of the kidney epithelium might be expected on diets deficient in vitamin A. van Leersum (100) has demonstrated the effect in rats by histological studies. Epithelial cells impregnated with calcium were found in the lumen of the tubules. Osborne and Mendel (78) had previously observed calculosis in the bladders of rats on diets deficient in vitamin A. Vitamin A deficiency probably played no part in the nephrotoxic effect of the more carefully designed experimental diets, which included adequate amounts of this substance. The calcium deposits in the tubules of the kidney described by van Leersum (100) are similar to the changes produced in the rat by the fat-free diets of Burr and Burr (15, 19). It is unlikely that any of the nephrotoxic diets employed in the past in rat experimentation were sufficiently fat-free to produce the deficiency disease described by Burr and Burr. In the discussion to follow it becomes apparent that vitamin deficiency was often present in conditions which led to kidney and blood vessel changes, but it is generally impossible to decide which known vitamin or vitamins may have been lacking.

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It is now established by the work of Yuasa (102) and of Schönheimer (89) that changes in the intima of the aorta and other blood vessels indistinguishable from the lesions observed in human arteriosclerosis may be produced in herbivorous or carnivorous animals by the addition of cholesterol to apparently adequate diets. The early work has been reviewed by MacCallum (52). Experimental proof was lacking in many of the earlier investigations because natural foodstuffs containing cholesterol instead of the purified sterol were used in the experimental diet. Since these natural foodstuffs were in many instances also high in protein, protein was cited as a possible causal factor. Later, when it was shown that cholesterol alone could be responsible for the changes produced, the question arose as to whether the arteriosclerosis produced in certain high protein feeding experiments could not be accounted for by the cholesterol content instead of the protein. Clarkson and Newburgh (23) showed in working with rabbits that when cholesterol was added to their control diet in amounts equalling the cholesterol content of their beef muscle diet, which produced arterial changes indistinguishable from the "cholesterol" sclerosis in rabbits, no changes were observed even though the duration of the feeding exceeded the time limit in which changes were produced by the high protein diet. It was only by increasing the added cholesterol tenfold that the characteristic lesions appeared. Anitschkow (5), however, fed rabbits only a few milligrams of cholesterol<sup>1</sup> (as milk) daily over a long period (2.5 years) and produced marked sclerosis, while Kon<sup>2</sup> failed to produce sclerosis in rabbits on a liver diet freed from cholesterol. It remains a fact that arteriosclerosis has never been produced in experimental animals by cholesterol-free diets. The arteriopathic, supposedly cholesterol-free, oats diet employed by Nuzum *et al.* (76) was augmented with cod liver oil, and the casein diet of Newburgh (69), which produced changes similar to those described by Nuzum, contained milk. The evidence at hand indicates that cholesterol might be the predisposing factor in all experiments in which arteriosclerosis has been produced; the cholesterol feeding experiments of Newburgh might indicate that the amounts necessary to produce the lesions are not independent of other diet factors. It should be noted that the control diet employed by Newburgh was markedly different from the arteriopathic diet in constituents other than cholesterol.

The significance of the ease with which cholesterol sclerosis is produced in the herbivorous rabbit in relation to the etiology of human arterio-

<sup>1</sup> The original paper gives no data for estimating the cholesterol ingested. The quotations of later reviewers may be based upon private communications.

<sup>2</sup> This reference was not found.



sclerosis is diminished by the results obtained in experiments with omnivorous animals. In these, arterial changes are produced only by massive doses and secondarily to a cholesterolemia and a large accumulation of lipid material in the other organs of the body, especially in the liver, a condition not characteristic of human sclerosis. Moreover, arterial or glomerular changes in the kidney comparable to the lesions associated with late human arteriosclerosis have not been produced by feeding excessive amounts of cholesterol. In the rabbit the kidney becomes fatty, but the changes resemble more nearly those in the spontaneous nephritis of rabbits. Evidence of a hypercholesterolemia in humans suffering with hypertension and arteriosclerosis has been sought, Pribram and Klein (82), Mjassnikow (63), and Labbé and Heitz (43) reporting positive results. Characteristic abnormalities in the blood lipids of patients suffering with these diseases were not found by Bloor (14), by Denis (28), or by Cantieri (21). The negative evidence must be given preference since a variety of abnormalities in the blood of arteriosclerotics has been reported, none of which is, however, characteristic. Negative evidence by no means settles the question. Bloor believes that the deposition of cholesterol and its esters does not necessarily require a high blood cholesterol, but that it depends on the ability of the blood to keep in solution a substance which is probably in a state of supersaturation.

The Virchow-Aschoff theory of the production of human arteriosclerosis through cholesterol deposition derives its greatest support from studies on diabetics, in whom the incidence of arteriosclerosis is unusually high and in whom lipemia is a characteristic condition in untreated cases (41). The lipemia of diabetes cannot be ascribed to fat feeding. Joslin has reviewed the cases described in the literature, where on improvement with high fat diets marked lipemias fell to normal levels. Insulin therapy has increased the death rate due to arteriosclerosis presumably by decreasing the deaths from coma. If hypercholesterolemia is the predisposing factor, insulin therapy would supposedly check the sclerosis in regulating fat metabolism by its established action in abolishing hypercholesterolemia. Bowen and Koenig (16) believe that neglect in treatment of diabetes leads to sclerosis. A complicating factor in studying the problem has been the use of high fat diets, incidentally high in cholesterol, in the treatment of diabetes. The widespread use of high carbohydrate diets as advocated by Sansum *et al.* (87) and by Adlersberg and Porges (3) will eventually furnish statistical data of a decisive nature. Joslin (41) compared the blood cholesterol values of groups of diabetics with either slight or marked arteriosclerosis with the values of a group free from sclerosis. The latter

group showed the higher values! Since the etiology of diabetes is of an unknown nature, and since the diabetic of the past was forced to subsist on an excessively high fat diet, the evidence gathered from the study of diabetes that a derangement in cholesterol metabolism might be responsible for arteriosclerosis loses force when applied to the non-diabetic arteriosclerotic. It has been impossible from statistical studies on the incidence of arteriosclerosis in meat-eating and non-meat-eating peoples to correlate cholesterol intake with incidence of arteriosclerosis, because those who do not eat meat usually consume large quantities of milk and eggs.

Because of the similarity in structure between cholesterol and vitamin D, the experiments on vitamin D overdosage, which result in marked histologic changes of the kidney and blood vessels accompanied by albuminuria and nitrogen retention, are of interest. The process is regarded as one of calcification. Calcium deposits have been observed in the aorta, (97a) and in the experiments of Light, Miller and Frey (48) the ash content of the kidney was increased sevenfold after vitamin D overdosage. These workers observed that the overdosage in the first and second generations produced striking pathological changes in the third and fourth generations. Moreau *et al.* (65) believe that the arterial changes resemble those of human arteriosclerosis, while Herzenberg (36) can see no similarity. A compromise is struck in the interpretation of Spies and Glover (94):

The composite histological changes (rabbit kidney) were strikingly different from the picture of any known pathological process occurring in man. Nevertheless, after decalcification, the lesions in the glomerular arterioles and the interlobular arteries did resemble the renal lesions associated with hypertension.

Schönheimer (89) has built up a very convincing picture of sterol metabolism, of particular interest in relation to the arterial changes which may be produced by cholesterol and vitamin D. He has shown that with the exception of irradiated ergosterol the phytosterols are not absorbed to any extent through the gastro-intestinal tract of either herbivorous or omnivorous animals, and that the presence of cholesterol in the body of the herbivorous animal and to some extent of the omnivorous animal must be accounted for by endogenous reactions. He has suggested that cholesterol is the starting point for an auto-oxidation reduction in which ergosterol and dihydrocholesterol are the end products.

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That kidney damage might result from the long continued excretion of excessive amounts of the end products of protein metabolism is not an unreasonable postulate, even on purely theoretical grounds. In 1919,

Newburgh (69) reported the production of nephritis in rabbits fed diets excessively high in protein, precipitating a controversy which is to-day not entirely settled. A review through 1928 by Mitchell appeared in an editorial in this Journal (62) and no attempt will be made to discuss all the experiments of that period. In general the criticism launched against experiments of a positive nature was the failure to demonstrate the presence of the nutritive factors other than protein essential to normal body development and function. Much discussion arose as to the identity of the histological changes observed with spontaneous changes known to occur, and as to the interpretation of histological findings.

Casein has been the protein most often employed in making up quasi synthetic diets for high protein feeding experiments. In 1925, Evans and Risley (31) reported tubular and glomerular kidney changes in rats fed high casein diets. Osborne and Mendel (78) had previously maintained rats on high casein diets for periods covering the seven-month period studied by Evans and Risley without producing kidney change. In 1926, Jackson and Riggs (40), Smith and Jones (91), and Addis and the MacKays (1), employing casein as the protein element of the diet, all reported negative results. The following year, Smith, Moise, and Jones (92), working with unilaterally nephrectomized animals, succeeded in producing tubular changes. In 1928, Newburgh and Curtis (71) compared the effects of a casein, a liver, and a muscle diet, and showed that kidney changes occurred more slowly in the casein than in the muscle and liver series, the minimum time set for casein being a year. On the basis of these results, the negative findings of certain of the earlier workers were invalidated, but the failure of Jackson and Riggs (40), who carried some of their experiments through eighteen months, to produce changes was still unexplained. At this time Moise and Smith (64), and Smith, Moise and Jones (92), showed that the age of the animal was a factor which had not been considered. Working with unilaterally nephrectomized animals, they found that no kidney changes were produced when the animals were placed on the experimental diets at thirty days of age for a period extending to 280 days, but that when the experiment was initiated on 120 to 200 day old rats, changes resembling the focal lesions that are found in old rats developed. In experiments of a similar nature, Jackson and Moore (39) obtained severe nephritis in four out of eleven rats. In the latest report of the Yale workers (79), gestation and lactation were superimposed upon the unilaterally nephrectomized rats. It was found that the diet employed by Smith, Moise and Jones (92) was not adequate for the production of good litters. More complex diets were instituted. Renal lesions were ob-

served in a few of the rats that were subjected to the greatest reproductive strain on a high casein diet in a three to six-month period. Barring experiments on very old or unilaterally nephrectomized rats, Newburgh and Curtis (71) and Evans and Risley (31) are the only workers reporting severe kidney damage on high casein diets in normal animals. The diets employed by Evans and Risley were obviously deficient in the elements necessary for normal growth. The question arises whether the diet employed by Newburgh, which was adequate for growth, did not lack other requisites for which there was no objective measure. The Newburgh diet contained 75 per cent casein, 16 per cent lard, 1 per cent salt, 3 per cent cod liver oil. Fifty milligrams of a yeast extract were given daily.

In summing up the work (on high casein diets), it may be concluded that on a high casein diet containing the elements essential to growth and normal function, rats are able to survive for periods covering the greater part of the normal life span without suffering renal lesions characteristic of nephritis.

Renal and arterial changes associated with the clinical pictures of nephritis and hypertension have been more readily produced by the use of diets containing natural foods than by those of a more synthetic nature. The nephropathic diets containing egg white, or soya beans, used in the original rabbit experiment of Newburgh (69), were obviously deficient in vitamin and salt content. The soya bean and peanut diets used by Evans and Risley were of the same category. The beef muscle and liver diets of Newburgh (71), used in his rat experiments, were augmented with salts, cod liver oil and yeast extract. Maclean *et al.* (55) believe that the kidney changes produced by the latter diet can be attributed to a lack of greens in the diet. These authors showed that marked tubular damage of the kidneys of rabbits fed on either high or low protein was produced in a month's time. The effect was abolished by the addition of a "small" amount of greens daily. Unfortunately the normal kidneys were obtained from animals fed on the greens diet only five to twelve months. A period of two years was required by Nuzum, Osborne, and Sansum (76) to produce both arterial and tubular and glomerular changes in rabbits on liver and oats diets augmented with cod liver oil and salts, and with greens given twice weekly. The beef protein diets of Newburgh (70) used in his rabbit series were augmented with 100 gm. of greens weekly. A discrepancy between the results obtained by Anderson (4) and those of Newburgh and Clarkson (70) is a stumbling block to interpretation. The diets employed by both workers were very similar. Beef and flour constituted the bulk of the diets, Newburgh's being augmented with 100 gm. of greens, and

Anderson's with 10 per cent alfalfa, and lettuce given once a week. Anderson superimposed a partial removal of kidney substance. No kidney damage was observed by Anderson while profound changes were reported by Newburgh. The Santa Barbara workers fed rabbits on barley for a 16 to 23-month period. The animals showed marked symptoms of diet deficiency, finally succumbing. Alfalfa was fed, 20 gm. weekly, after the first six months of the experimental period. No kidney or blood vessel changes were noted. The experiments by Maclean (55) have been cited so extensively that it seems fitting to consider them in some detail. Their low (12 per cent) protein diet consisted of bran and oats. Their high (60 per cent) protein diet contained bran, casein, gelatin and salt. Both of these diets produced tubular lesion in less than two months. The addition of "a little green food" entirely prevented the occurrence of kidney damage. While the actual amount of green food is not given, it transpires that two leaves of cabbage a day were fed each rabbit. It is also noted that the reaction of the urine on the cabbage diet was with one exception alkaline or neutral, while that of the greens-free diet was acid. Since rabbit kidneys do not compensate to any great extent in the formation of ammonia when acid urine is being produced, it is difficult to understand how the daily ingestion of only two leaves of cabbage shifted the reaction of the urine so markedly. Maclean's work undoubtedly showed that the tubular lesions which developed in the rabbits on his particular greens-free diet cannot be traced to the dietary protein.

Since high casein synthetic diets produce kidney lesions only under exceptional strain, and certain natural food protein diets such as liver produce renal damage in a comparatively short time, it is more or less evident that if protein is the predisposing factor, the nephropathic property is not common to all proteins. Mitchell (62) has emphasized that the production of renal changes might just as well be attributed to the non-protein constituents of the natural protein foods. Newburgh and Johnston (73) recently described experiments corroborating these views. They found that high protein feeding of lactalbumin, and of wheat and soya bean gluteins, augmented with vitamins and salts, produced no renal changes in a year's time, while a 40 per cent liver diet, or a 20 per cent sodium nucleate diet, was nephrotoxic in less than a year. The nucleic acid was prepared from liver, and when ingested in the amounts contained in a nephropathic liver diet was insufficient to produce renal changes. The nephropathy produced by nucleic acid was characterized by formation of scar tissue and by thickening of the arteriole walls, a picture not the same as that produced by liver feeding. Newburgh concluded that liver may contain

nephrotoxic forms of non-protein nitrogen other than purines. The deduction is not clear since the nucleic acid was presumably bound in a protein molecule in the liver, and would consequently be considered an intermediary product of protein metabolism. The recent preliminary report of Blatherwick *et al.* (13) on the effect of liver and beef muscle diets on unilaterally nephrectomized rats will leave no doubt to even the most skeptical histologist that lesions of great severity, very similar to chronic glomerulonephritis in man, may be produced by feeding experiments. Blatherwick supplemented his diets with yeast, cod liver oil, lard and salts, and also greens, so that the objections raised to older work are met. Blatherwick, like Newburgh, also studied a liver residue fraction with the idea of finding what fraction of liver contains the nephrotoxic substances.

The production of renal injuries by the intravenous or oral administration of certain amino-acids, notably cystine, has been cited as evidence that high protein feeding is one of the factors in the etiology of nephritis. An obvious objection to experiments in which cystine was fed as an amino-acid is that free cystine would not suffer the same ultimate fate as protein-bound cystine because of the difference in the rate of alimentary absorption. Curtis, Newburgh, and Thomas (27) established the amounts of cystine which would injure the rat kidney. A diet containing 1.5 per cent cystine produced necrosis of the tubules in one year, while 5 per cent cystine was lethal in a few weeks. Evidence has recently been presented by Cox which indicates that, at least as far as young animals are concerned, vitamin B requirement and cystine intake are closely related. Cox and Hudson (25) showed that the addition of large amounts of yeast to a 0.3 per cent cystine diet, which was nephrotoxic to young rats, prevented the renal injury entirely. It seems worth mentioning that the 1 per cent cystine diet fed rats by Addis (1), without production of renal changes, contained 10 per cent yeast. Unfortunately Addis stopped his experiments at twelve months, the minimum time Newburgh found necessary to produce lesions of the kidney on a diet containing approximately the same amount of cystine, so that it remains to be shown whether or not the protective effect of yeast against the nephropathic action of cystine is the same in young and old animals. When Hartwell (34) substituted edestin for casein in feeding experiments on young rats, the animals died showing kidney changes, unless vitamin B content was greatly augmented. The protective factor was not destroyed by autoclaving. Older rats were not affected by the diet low in vitamin B. Hartwell concluded that edestin requires more yeast than either caseinogen or egg albumin for normal metabolism. Unfortunately a histological report of the pathological kidneys described by

Hartwell is not available. The experiments of Cox and of Hartwell indicate that the minimal vitamin B requirement is dependent on the nature of the other diet constituents. If this postulate be accepted, the interpretation of all diet experiments concerned with renal changes is complicated by the uncertainty of the vitamin B requirement, even the recent work of Newburgh (73) and of Blatherwick (13). Francis, Smith, and Moise (32) have recently shown that the renal hypertrophy caused by the feeding of high protein diets is not affected by vitamin feeding. Their experiments did not produce kidney damage. There is at present no evidence that the excretion of any of the known amino-acids in the human is responsible for renal injury. Excepting conditions in which there is severe liver damage, the amino-acid concentration of the blood never rises markedly and relatively small amounts of amino-acids appear in the urine.

Realizing the inherent difficulties involved in designing a diet experiment for laboratory animals which will produce decisive evidence, one approaches the human experiment with great temerity. Following the results of high protein feeding on animals, Squier and Newburgh (95) studied the effect of high protein feeding in normal humans and in several cases of essential hypertension. When two normal men ate three pounds of steak a day for several days, red cells but no albumin appeared in the urine. In five cases of essential hypertension, the ingestion of 100 to 175 gm. of protein per day over a period of two to twenty-one days was accompanied by the appearance of red cells and albumin in the urine. It is now generally recognized that the appearance of red cells in the urine under the experimental conditions of the Squier-Newburgh experiment is without significance. McClellan and DuBois (50) studied the effect of a meat diet containing 100 to 140 gm. of protein and 200 to 300 gm. of fat, which was followed for a year. No evidence of kidney damage was obtained. Nitrogen equilibrium was maintained on a daily intake of 19 gm. Newburgh, Falcon-Lesses, and Johnston (72) repeated the high protein experiment, using a diet containing 337 gm. of protein per day, 31 per cent of the caloric intake being in protein. Albumin and casts appeared in the urine of a normal individual who followed this diet for a six-month period. On resuming a normal diet, the albumin and casts disappeared. It is controversial whether the appearance of a slight amount of albumin and a few casts is indicative of renal damage. In the rabbit, the appearance of albumin follows uniformly the excretion of an acid urine. In the experiments of Bischoff (12) no histological renal changes were found in rabbits which had passed an acid urine and shown traces of albumin for nearly two years. The albuminuria produced in rabbits by the passage of

an acid urine has been explained on the basis of a change in cell permeability.<sup>3</sup> The Newburgh diet (72), as far as protein content is concerned, approached more nearly the diet of the Eskimo near civilization while that of McClellan and DuBois (50) was similar to the diet of the Eskimo away from civilization. The Eskimo, subsisting on a carnivorous diet, which according to Krogh averages 280 gm. of protein daily, ingests excessive amounts of cholesterol, protein, and acid ash, and apparently tolerates these supposedly harmful substances well, for the incidence of renal-cardiovascular diseases according to Thomas (99) is low.

Newburgh (72), however, is inclined to discount the conclusions of Thomas, based on a survey of 142 Greenland Eskimos, that an 8.5 per cent incidence of albuminuria was not indicative of prevalent renal disorders. He cites the statistics on over 16,000 policy-holders of the Metropolitan Life Insurance Company. The comparison is hardly justifiable since policy-holders are picked. Moreover, the age limit of the group of policy-holders selected was five years below that of the group of Eskimos. Furthermore, the difference in incidence of albuminuria for the two groups is less than three times the standard deviation of the mean. The discrepancy in results obtained in the Newburgh experiment, in which albuminuria was produced, and the findings in the studies on the Eskimo, who does not normally show albuminuria, has not been explained. The Newburgh diet contained 80 gm. of liver a day. It is regrettable that this substance, which according to Newburgh's own animal experiments has a specific nephrotoxic effect, was included in the diet.

The experiments of McClellan and DuBois indicate that amounts of protein which would be considered high in the diet of civilized man may be eaten for a comparatively long period without suggestion of renal injury. The Newburgh experiments indicate that the tolerance of the human kidney to certain high protein food metabolites is not without limit.

It should be emphasized that Newburgh (72, 95) noted no effect of high protein feeding on blood pressure, an observation in agreement with the reports of Strouse and Kelman (97) and of others, that variations in blood pressure bear no direct relation to intake of protein. The latter statement is, however, not universally accepted. The low incidence of hypertension among the Chinese has been ascribed to the diet, which is low in protein. Cadbury (20) found that the blood pressure of Chinese was lower than that of Americans or Europeans of the same age. Diet is apparently not the only factor, for according to Houston (37a) most white

<sup>3</sup> Bayer, W., *Z. ges. exper. med.*, 1928, 59, 162.



men have a fall in blood pressure during a prolonged sojourn in China, even on a normal European diet. Saile (85), in comparing monks who subsisted upon an herbivorous diet with those who partook of meat, found a lower blood pressure range in the group on the vegetarian diet. It should be noted that the vegetarian monks partook of only bread and water on their weekly fast day and during their forty-day fast period. In contrast to the meat-eating groups, the vegetarian monks were allowed very little liquor. Underfeeding has been shown by Benedict *et al.* (11) to lower blood pressure. The subjects observed by Cadbury (20) and by Saile (85) may have been undernourished. Mosenthal (66) originally suggested the latter explanation to account for the impression that low protein diets lower blood pressure.

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Since many of the synthetic and natural food high protein diets, associated with the production of renal and blood vessel changes, were necessarily high in "acid ash," the nature and content of the ash became one of the variables to be considered in seeking the causal factor. Such diets produce an acid urine, and the proof of the theory lies in showing that the degree of acidity in itself, or the burden placed upon the kidney in maintaining the acid base equilibrium of the body, is responsible for the renal changes. In order to show a relation to the production of arteriosclerosis, a demonstration of a shift in the acid base equilibrium of the blood or tissues would be required. Newburgh and Clarkson (70) in their original rabbit experiments attributed the renal injuries produced either to the excessive excretion of amino-acids or to a combined effect of amino-acids and acid urine. They did not consider the acidity of the urine by itself capable of producing all the injury. Nuzum, Osborne and Sansum (76), using more complete diets and instituting a series of controls, confirmed the work of Newburgh (70), but hesitated to ascribe the results to the protein *per se*. Since they observed a decrease in the CO<sub>2</sub> combining power of the blood plasma of rabbits maintained on oats or liver-containing diets, they had produced evidence that acidosis might be a predisposing factor. In later experiments, however, Nuzum *et al.* (77) failed to reproduce the fall in CO<sub>2</sub> combining power on the same diet used in the earlier experiments in spite of the fact that animals with a spontaneous nephritis were used. The changes observed, other than the fall in CO<sub>2</sub> combining power, confirmed the original work. Bischoff, Sansum, Long, and Evans (12), using a diet of the same acid ash content as that employed by Nuzum and instituting a more refined technic in the determination of acid base changes, failed to observe changes in plasma pH and CO<sub>2</sub> content, or

damage to the blood vessels or kidneys of rabbits. Nuzum and Newburgh both have produced by an alkaline ash soya bean diet, kidney changes similar to those produced by acid ash diets. The original nephropathic casein diet of Newburgh was potentially neutral in reaction. It would therefore appear that in the rabbit the association of acid ash with kidney and blood vessel changes was fortuitous.

The kidney of the rat has such a remarkable compensating mechanism in the formation of ammonia when the urinary constituents are predominantly acid in nature, that acid urines down to the danger point of pH 4.8 can not be produced by feeding acid ash foodstuffs. The lowest value found by Long and Sansum (unpublished data) for acid ash foodstuffs was 6.1. In order to eliminate the increased burden inflicted upon the kidney in ammonia formation, Polvogt, McCollum, and Simmonds (81) adjusted their high protein diets so that the ash content was potentially alkaline. They observed renal changes. Addis (1) added calcium chloride to his casein diet so that the urinary pH was 5.2 as compared with 6.4 for his control series. No kidney damage was produced. Recently Blatherwick (13), working with unilaterally nephrectomized rats, produced nephritis by liver feeding. In one series of his experiments, sodium bicarbonate sufficient to neutralize the acid ash of the liver was added. The kidney damage, if anything, was greater on the neutral ash diet. The conclusions recently formulated by Samuel and Kugelmass (86), that acid-forming diets shift the acid base mechanism and inhibit growth, development, and metabolism in young rats, are obviously unwarranted, since the acid base factor was only one of many variables in their two series of experiments. These investigators ignored the work of Green and Mellanby (33) on the interfering effect of cereals on calcification, although their acid ash diet consisted of egg, rice and oatmeal. Their basic diet contained dried milk, potato, and beans.

In man the acid or alkali content of ingested foodstuffs is insufficient to produce a sustained shift of the acid base equilibrium outside the limits of normal. Michalowsky (60) observed no changes in the alkali reserve on a mixed diet of meat, fat, and wheat. Unpublished data of Bischoff, Long and Sansum show that the daily ingestion by an arteriosclerotic patient of sodium citrate equivalent to 18 pounds of oranges for a period of three months, shifted the pre-breakfast plasma pH and total  $\text{CO}_2$  of the blood just significantly outside the individual's control range, but not outside the normal group limits. The ingestion for a three-day period of protein foods containing an acid ash equivalent of 2.5 pounds of beefsteak failed to produce any effect on the acid base equilibrium of the blood, although

there was marked nitrogen retention. The urinary pH failed to become more acid than 5.0 due to the increased formation of ammonia. Previously Michalowsky (60) had shown that the ingestion of 2.5 gm. of phosphoric acid daily (equivalent in acid ash to less than one pound lean beef) for a period of six weeks had no effect upon the alkali reserve. It is likely that the ingestion of natural foodstuffs produces temporary changes in the acid base equilibrium of the blood, but it is doubtful whether these changes are as great as those produced by exercise, and, therefore, whether they are significant. Experimentation is complicated by the "alkaline" tide and awaits investigation.

While there is at present little evidence that the acid ash foods produce kidney or blood vessel changes by virtue of a shift in the acid base equilibrium of the body, it is possible that the character of the ash constituents, other than potential acidity, is involved in the changes produced. Diets high in the acid ash foods are usually high in phosphorus and low in calcium. The calcium-phosphorus ratio has been adjusted in the nephropathic diets of a sufficient number of workers (Nuzum (76), McCollum (81), Blatherwick (13)), to eliminate the factor of ratio. A high phosphorus content, irrespective of calcium low or high, has not been controlled. The failure of many workers to produce renal damage in rats on high casein diets and the failure of the Santa Barbara workers to produce renal changes in rabbits on prolonged barley feeding, involving in both cases high phosphorus diets, is evidence of a negative character that phosphorus is not a causal factor. MacKay and Oliver (53) have assigned a positive influence to phosphates in affecting renal hypertrophy and tubular damage. They found that acid, neutral and basic phosphate, all produced the same effect, so that the reaction of the phosphate was eliminated as a factor. Deductions concerning the effect of high phosphorus (high protein) diets based on the feeding of phosphate are analogous to deductions concerning the effect of high protein diets based on the feeding of amino-acids; the liberation of bound phosphorus as phosphate in the catabolism of ingested nutrients would presumably be a more prolonged and uniform process than the absorption of phosphate. Constituents other than calcium or phosphorus in the ash content of the diet have thus far not been considered in feeding experiments concerned with the production of renal or blood vessel changes.

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In summing up the experimental work on the effect of diet on renal and blood vessel changes, it becomes apparent that changes in the blood vessels outside the kidney, and changes in the tubules, glomeruli, and ar-

terioles of the kidney, may be produced by diet constituents, but in no case can any one nutritional entity be held responsible for producing a clinical picture resembling the progressive characteristics of human hypertension followed by arteriosclerosis and by nephritis. Newburgh (70) and Nuzum (76) came close to producing such clinical pictures in rabbit experiments, but three variables at least may have been concerned in producing the changes: cholesterol, specific protein foods, and lack of vitamins. The cholesterol might account for the arterial changes and the protein or associated compounds for the renal injury, while vitamin deficiency with attending bacterial invasion can not be eliminated as a superimposed influence. In rat experiments, no aortic changes were produced by nephrotoxic diets, and no characteristic renal changes by arteriopathic diets. In rats, Blatherwick (13) produced albuminuria with a change in the ratio of the plasma protein by feeding natural protein foods, the very substances used in the treatment of humans with the same findings. The original ideas concerning the three constituents which have been held responsible for kidney or blood vessel changes induced by diet feeding experiments, have not stood the test of time. Sensitivity to cholesterol has been shown to vary largely with species. The ideas concerning the effects of high protein feeding have undergone a series of metamorphoses; the effects ascribed to protein are either specific for a certain kind of protein or are due to non-protein constituents associated with protein foods. Acid ash at present appears to be without significance. In the case of humans,<sup>4</sup> degenerative changes produced by lack of vitamin A in the diet or by an excess of vitamin D in the diet would presumably be regarded as isolated cases. In considering the rôle of diet in the treatment of arteriosclerosis and nephritis, the greatest caution must obviously be exercised in drawing deductions from animal experiments. If man were to eliminate from his diet all the substances which have been held responsible for producing blood vessel and kidney changes in animal experiments, he would become a vegetarian in the strictest sense of the word, denying himself even milk or eggs. Incidentally, in joining the herbivora he would be joining a class with a high incidence of kidney damage.

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The nephritic of twenty years ago was invariably placed upon a low protein diet. Restriction of nitrogen intake was considered desirable to lessen the burden upon the kidney in excreting urea. The practice, however, anteceded this explanation of the last generation. It is possible that the

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<sup>4</sup> Harris, L. J., *Annual Rev. Biochem. Stanford*, 1932, 1, 365.

production of albuminuria was associated with a misconception of a derangement in protein metabolism prompted by the analogy of sugar excretion in diabetes. It is now generally recognized that the nephritic is no exception to the rule that the protein requirements of the organism must be met, and since there is in nephrosis and certain other types of Bright's disease a considerable leakage of protein in the urine, the protein requirement may be greater than that of the normal individual. Epstein (30a) originally advised the use of high protein diets for certain types of nephrosis. Peters and Van Slyke (80) have emphasized the dangers of low protein feeding in nephritis. These authors point out that there is no relation between the amounts of protein fed and the amount excreted in the urine on moderately high protein diets; furthermore adults with the degenerative type of nephritis may apparently be protein-starved, since they have an amazing ability to store nitrogen for long periods without increasing the non-protein nitrogen of the blood or urine. Although the non-protein nitrogen of the blood of patients with advanced nephritis may be reduced by low protein-feeding, Peters and Van Slyke doubt the significance of this finding in relation to the cause of the disease. The clinician is thus faced with a paradoxical situation. Although animal experimentation has shown that high intake of certain protein foods is productive of renal injury, he must discount the probability of increased consumption of nephrotoxic substances for the more urgent requirements of the body as a whole.

In a study of 500 persons, Langstroth (45) found an apparent relation between the percentage of non-protective (vitamin deficient) food which had made up the usual diet of the subject and the incidence of degenerative disease. On the basis of this observation, forty odd patients with chronic circulatory diseases were placed on diets high in protective foods. Marked improvement was noted. A significant fall in blood pressure was observed in 18 out of 23. Langstroth attributed the effect in part to a reduction in caloric intake, a procedure which previously had been found effective by Rose (84) and by Terry (98), but emphasized the importance of a high intake of protective foods. He pointed out that the fall in blood pressure did not in all cases occur independently of relief from distressing symptoms. Langstroth's dietary survey was based on information obtained by questioning the subjects, and is in this respect unique. The results are certainly in harmony with animal experimentation, in which degenerative changes are more easily produced on vitamin-deficient diets. The clinical results reported by Langstroth are typical of those attributed to scores of regimes for the treatment of circulatory diseases, but proof is lacking that the regime affects or even arrests the degenerative processes.

The fundamental difficulty in work of this nature is the lack of acceptable objective measures. A fall in blood pressure unfortunately can not be regarded as convincing, since the blood pressure of the patient with hypertension is notoriously subject to great lability. Ayman (7) recently prescribed "seriously and enthusiastically" to forty unselected hypertensive patients a daily dose of a few drops of very dilute hydrochloric acid and found definite improvement in 82 per cent of the cases. He concluded that the symptoms associated with uncomplicated essential hypertension<sup>5</sup> may frequently be relieved by the suggestion inherent in a prescribed method of treatment. Since the non-protective foods are as a class high in acid ash, the alkaline ash diets of Sansum *et al.* (88), used in the treatment of vascular diseases, are essentially the same as those employed by Langstroth, although the *modus operandi*, the production of a neutral urine, is of course entirely different. A diet along the same lines had previously been described for the treatment of nephritis by Chace and Rose (22). What has been said about the Langstroth diet applies equally well to the diets of Sansum and of Chace. A statistical survey of survival rates of patients with essential hypertension who have followed such diet regimes as compared with patients who were allowed freedom in food selection, would do much to settle the question.

Since a loss of base with an attending acidosis may be a manifestation of terminal nephritis, the use of alkaline ash diets in the treatment of nephritis would appear logical. The objection to these diets is that they are apt to be too low in protein. Nothing is gained in attempting to neutralize the sulfur and phosphorus of protein by the addition of fruits and vegetables high in sodium and potassium, since the work of Shohl and Sato (90), of Briggs (17), and of Bassett, Elden, and McCann (9), show that the elimination of the acidic elements is not enhanced by increase of base. According to Briggs, the virtue of the basic diet lies in the limitation of the phosphoric and sulfuric acids to be excreted and the specific action of calcium in increasing elimination of those acids. While it is recognized

<sup>5</sup> It may be questioned whether essential hypertension is properly included under the heading of kidney and blood vessel changes. Even to-day it is not universally recognized that hypertension generally precedes arteriosclerosis by a considerable lapse of time. In the experiments of Nuzum *et al.* (76, 77) and of Anderson (4) discussed in this review, blood pressure data were collected. Anderson noted no change in the blood pressure of the rabbits which developed arteriosclerosis. Nuzum on the other hand noted a slight elevation in blood pressure for the groups of rabbits which developed arteriosclerosis, but none for a group which showed kidney damage without arteriosclerosis. The production by diet experimentation of arteriosclerosis, the lesion of significance in human pathology unless associated with a preceding hypertension, may be an entirely different process from that taking place in humans.

that the ability of the kidney for ammonia formation is reduced in nephritis, there is disagreement as to whether ammonia formation conserves base or merely protects the kidney from acid. Whatever the mechanism, the basic diet would appear superior to one high in acid ash, provided the protein is adequate. Peters (80), admitting the basis for the use of alkaline ash diets in terminal nephritis as theoretically sound, has pointed out that experimental data are lacking to show the effects of the treatment.

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The survey of animal experiments, of diet habits of various peoples, and of clinical diet procedures, leaves one with the impression that diet may have little to do with the spontaneous kidney and blood vessel changes observed in lower animals and with the cardiovascular renal diseases found in man. The progress of the research is at that stage where positive results are attained only by a degree of exaggeration which destroys their practical significance. A more thorough application of the statistical method and more carefully designed clinical experiments based on acceptable objective measures must be instituted in the study of diet in hypertension, arteriosclerosis, and nephritis. The uncertainty of the effect of diet on blood vessel and renal changes in man is an unwelcome spectre in the sciences of nutrition and medicine.

FRITZ BISCHOFF

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THE EFFECT OF A HIGH INTAKE OF MANGANESE  
ON THE GROWTH OF RATS\*

BY J. T. SKINNER

*(From the Department of Agricultural Chemistry,  
University of Wisconsin, Madison)*

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**I**N A previous paper from this laboratory (1) it was reported that young rats receiving a modified stock ration plus manganese at high levels grew less rapidly than did similar animals which received the regular stock ration without manganese additions. No definite conclusions could be drawn from these results because, besides manganese, another variable was inadvertently introduced into the ration. For the purpose of better controlling the intake of added manganese the milk which had been fed *ad libitum* to the control group had been replaced in the case of the manganese-fed animals by an approximately equivalent amount of milk powder. That the slower growth was due to a less suitable source of milk solids rather than to a toxic effect of manganese was indicated by the continued growth of the manganese-fed animals until at the end of 6 months their weights were almost equal to those of the controls. Some additional evidence that manganese was non-toxic at the level fed was furnished by the good growth obtained in some preliminary experiments with a ration containing liquid milk and added manganese.

That the ingestion of large amounts of manganese was responsible for this inhibition of growth was suggested by earlier reports from other laboratories. Richet, Gardner, and Goodbody (2) found that manganese decreased growth if given to dogs at the rate of 1 gm. per day, whereas it exerted a beneficial effect when given every third or fourth day. McCarrison (3), working with rats, found that growth was less rapid after the thirty-second day if 0.56 mg. of manganese as  $MnO_2$  was included in the animal's ration. Nelson, Evvard, and Sewell (4) reported a less efficient utilization of the ingested food as well as a slower rate of growth by rats on a ration containing 600 p.p.m. of  $MnSO_4 \cdot 4H_2O$  than upon the unsupplemented ration.

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In view of these somewhat confused and conflicting results it appeared desirable that a further study should be made of the effect of a high intake of manganese.

#### EXPERIMENTAL

The investigation may be divided conveniently into two parts 1.—experiments dealing with the pre-weaning period, when the effect upon the growth of the young is exerted indirectly, i.e., through the mother, and 2.—experiments covering the post-weaning period, when the effect is exerted directly upon the young.

The basal ration used throughout this investigation was that previously described by Waddell and Steenbock (5) and contained 13.4 mg. of manganese per kilo.

*Effect of Manganese Prior to Weaning.* In this study growth records were obtained on a number of animals from mothers receiving the stock ration with and without manganese additions. For controls six virgin females and one male were placed in a cage provided with shavings and fed the ration previously mentioned plus milk *ad libitum*. Another group of eight females and two males placed in two cages received the basal ration plus 10 mg. per animal daily of manganese as  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ . This large quantity of manganese, which is double that fed in the earlier experiments, was chosen with the intent to magnify any injurious effects that might come from the ingestion of this element.

Difficulty was immediately encountered in that the females of both groups showed a marked inability to rear their litters to normal size at weaning age. Moreover, an unusually high percentage of the young died. Since a similar trouble in the stock colony had been partially eliminated by feeding yeast, the ration of each group was modified by replacing a portion of it, three per cent, with brewers' dried yeast or its equivalent in fresh yeast. Although by these means the difficulty was largely removed, at no time during the experiment was growth in either group consistently equal to that ordinarily obtained on the stock ration. However, since the controls and manganese-fed animals were grown simultaneously, the results probably are as significant as those which would have been obtained under normal conditions.

Prior to parturition the females were placed in individual wire cages provided with shavings where they were kept until the young were weaned. In order that the young might have a proper chance for nourishment, the number was reduced to six per litter on the seventh day. Weights were obtained at birth and on the seventh, fourteenth, and twenty-first days thereafter. Although observations were made daily between the ages of

8 and 21 days, the data for convenience have been tabulated into three periods of seven days each, 1 to 7, 8 to 14, and 15 to 21 days, respectively.

Since one female in the manganese group failed to breed, the data on animals prior to weaning represent the performance of the young from six females in the control group and seven females in the group receiving manganese.

In Table I it will be observed that the young in the control group averaged 5.5 gm. at birth whereas the young from females receiving manganese weighed 5.8 gm. This lower weight in the former group was probably due to greater numbers of young per litter, the average being 8.4 as compared with 7.8 in each litter of the manganese group. In addition to those living at birth there were three animals in the control group and five in the manganese group which were born dead. None of these slight differences indicates a prenatal effect due to manganese.

During the first 7 days of life the group receiving manganese suffered less than half as many deaths, proportionately, as did the controls, 19.5 per cent as compared with 45.5 per cent. In the second period, however, the loss in this group was 60 per cent greater than in the control group. These marked differences were, to a great extent, due to the high death rates in the litters from two females, one in each group. Whereas the female belonging to the controls lost two of her three litters during the first period, the one in the manganese group lost the same number from as many litters during the second period. The differences in growth of the two groups during these two periods were quite small and are insignificant, if the number of animals per litter at the end of the periods be considered.

Although no deaths occurred in either group during the third period, the young reared by mothers receiving additional manganese attained a weight of 40.4 gm. by the twenty-first day as compared with an average of 37.0 gm. for the controls. This 9 per cent increase in weight together with a lower death rate during the first 7 days of life may not be significant, but at least these data prove quite conclusively that manganese in relatively high concentrations does not prevent female rats from nourishing their young properly. Hence the greater death rate and slower growth of animals which were noted in our previous work (1) must have been due to differences in the source of milk solids rather than to differences in manganese intake.

*Effect of Manganese from Weaning Age to Maturity.* For this experiment twenty male and twenty female rats were selected from nine litters of young, 23 to 26 days of age, and divided into two groups containing

TABLE I  
PERFORMANCE OF RATS PRIOR TO WEANING AGE AS INFLUENCED BY MANGANESE INTAKE OF DAMS

Manganese intake	Period	Number of litters	Number of young		Percentage of young lost	Ave. wt. of young	
			At beginning	At end		At beginning	At end
*Moderate level	I. 0-7 days	16	134	73	per cent 45.5 12.5 0	gm. 5.5 12.0 24.7	gm. 12.3 24.7 37.0
	II. 8-14 "	11	56	49			
	III. 15-21 "	11	49	49			
†High level	I. 0-7 days	19	149	120	19.5 20.0 0	5.8 12.0 26.2	11.6 26.2 40.4
	II. 8-14 "	14	75	60			
	III. 15-21 "	12	60	60			

\* A stock ration containing 13.4 mg. of Mn per kilo was fed, together with milk *ad libitum*.

† In addition to the stock ration and milk each adult female received 10 mg. of Mn daily.

TABLE II  
GROWTH OF RATS AFTER WEANING AS INFLUENCED BY MANGANESE INTAKE

Manganese intake	Number of animals	Sex	Original weight		Weight at end of 7 weeks		Weight at end of 12 weeks		Average daily consumption of dry ration
			Limits	Average	Limits	Average	Limits	Average	
•Moderate level	10	♂	gm.	gm.	gm.	gm.	gm.	gm.	gm.
	10	♀	45-54 45-54	50.2 47.6	235-293 162-211	255.1 200.0	309-352 201-259	334.8 226.9	13.2 8.4
†High level	10	♂	43-54	50.3	228-301	261.1	293-381	334.6	13.5
	10	♀	46-53	48.1	170-211	186.6	198-265	223.9	8.8

\* A stock ration containing 13.4 mg. of Mn per kilo. was fed together with milk *ad libitum*.

† In addition to the stock ration and milk each animal received 2 mg. of Mn per day during the first 7 weeks and 10 mg. per day thereafter.

equal numbers of both sexes. An effort was made to distribute the animals of a given litter so as to equalize weights and hemoglobin values.

Males and females of each group were placed in separate wire cages and provided with shavings. The stock ration previously mentioned was supplied in such amounts that consumption was barely exceeded and whole milk was provided *ad libitum*. No attempt was made to learn the amount of milk consumed. The consumption records for the dry ration, however, are fairly reliable since tin covers were placed over the feed jars to prevent wastage.

In addition to the stock ration and milk the males and females in one group received 2 mg. per animal daily of manganese as  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$  for the first 7 weeks and 10 mg. per day thereafter for 5 weeks, at the end of which time the experiment was terminated. These levels, which were twice as high as those fed in our earlier work (1), were expected to demonstrate more speedily and effectively any deleterious effects which might be due to the ingestion of large amounts of the element, manganese.

As shown in Table II, at the end of the first 7 weeks the average weight of the males receiving the additional manganese was 261 gm. as compared with 255 gm. for the controls. On the other hand, the females receiving manganese had not done so well, as was indicated by an average weight 13.4 gm. less than that of the females in the control group. During the remaining 5 weeks these differences were almost completely eliminated. Only 0.2 gm. difference in the average weight of the males in the two groups was found at the conclusion of the experiment, while the females in the manganese fed group averaged only 3 gm. smaller than those in the control group. Obviously the manganese intake had exerted no retarding effect upon growth.

Apparently there was little if any difference between the two groups in the efficiency with which the food was utilized, as is indicated by the average daily consumption shown in the last column of Table II. If these be considered as representing the daily food intakes—although some wastage unavoidably occurred—the manganese group consumed only 3 per cent more ration than did the control group. Thus growth and utilization of food were practically unaltered even though the manganese intakes were increased 31 and 48 times for males and females respectively.

Obviously these rats were fed much larger amounts of manganese than McCarrison (3) found necessary to retard growth. In order that the manganese intake could be accurately compared with that of the animals fed by Nelson, Evvard, and Sewell (4), the total amount of manganese added over the entire period has been calculated in terms of parts per million of

dry ration consumed. On this basis the males in the manganese group received, in addition to that occurring naturally in the ration, 1607 p.p.m. of  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$  and the females received 2465 p.p.m., the average for both sexes being approximately 2000 p.p.m. This is more than three times as high as the level at which Nelson and his associates fed their animals.

Hemoglobin determinations were made at the beginning of the experiment, at the end of 7 weeks, and at the termination of the experiment. The data obtained have not been detailed in a table since the experiment was designed for a study of the effect of manganese on growth and the results were of secondary interest to us. However, no appreciable differences were found which could be attributed to an effect of the added manganese upon either males or females. To illustrate, at the beginning the hemoglobin values for the males in the control group varied from 7.10 to 10.36 and averaged 8.7 gm. per hundred cc., while in the manganese group the limits were 6.06 and 10.94 with an average of 7.88 gm. per hundred cc. At the end of the experiment the values for the two groups were: 1.—controls—limits 13.49 to 17.05, average 15.30; 2.—manganese-fed—limits 13.20 to 17.05; average 15.85 gm. per hundred cc.

#### SUMMARY

Female rats which received 10 mg. of manganese per day above that in a stock ration (13.4 mg. per kilo) were as successful in rearing their young as females receiving the stock ration only.

The addition of  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$  equivalent to 2000 parts per million of ration did not retard the growth of rats over a period of 12 weeks immediately following weaning.

Therefore, the slower growth of rats on a modified ration containing added manganese, which was reported in a previous publication (1), was not due to toxicity of this element but to a less suitable source of milk solids. Since under different conditions high levels of manganese may retard growth, no attempt is made to explain the contrary results of others by the data presented in this paper.

The author wishes to express his thanks to Professors H. Steenbock and W. H. Peterson for their valuable suggestions.

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# THE DIETS OF COLLEGE WOMEN IN RELATION TO THEIR BASAL METABOLISM\*

By

CALLIE MAE COONS AND ANNA T. SCHIEFELBUSCH

*(From the Department of Agricultural Chemistry Research, Oklahoma Agricultural and Mechanical College, Stillwater)*

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## INTRODUCTION

IN THE effort to find satisfactory explanation for the low basal metabolism of "normal" women in Oklahoma (Coons, 3), the author pointed out that a comparison of the rates for selected groups indicated that nutrition, in addition to climate, might be a contributing factor. Quantitative dietary studies in conjunction with basal metabolism observations might be expected to throw some light on the problem. The analyses of the self-chosen diets of 18 individuals and of the experimental diets of two others are presented in this paper and discussed in relation to the basal metabolic rates.

## LITERATURE

Evidence has been accumulating to show definite marked differences in the basal metabolic rates of southern women as compared to those in the northern part of the United States. Women at Wellesley (Gustafson, 5), Columbia University (MacLeod, 7), and in Ohio (McKay, 8) are reported to have basal metabolic rates averaging 5 to 8 per cent below the Aub-DuBois standards, while very recently some in South Carolina (Remington, 9) and Florida (Tilt, 10) were found to average  $-10.4$  and  $-10.6$  respectively.

In the Oklahoma studies women 17 to 20 years of age had rates averaging around  $-14$ , Aub-DuBois, and those 20–29 years around  $-12$ , or an average of  $-13.2$  for all ages observed. Oklahoma has a climate less tropical than Florida, or even South Carolina, so that climate alone probably is not responsible for all of the lower rate.

The protein intake of students, chiefly men, from different sections of the United States has been found to be without great difference. Youngburg (12) reported for 12 individuals at Buffalo an average urinary nitrogen of 11.3 grams, equivalent to 76.6 grams protein intake per 24 hours for 70 kilo. men; Brooks (1) an average of 10.34 grams, 71.3 grams protein intake, for 192 male students in North Carolina; and Denis (4) 10.63

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grams nitrogen, 73.8 gram protein intake for 233 male students in New Orleans.

Wakeman and Hansen (11) have shown that the basal metabolic rate is lowered appreciably only after prolonged subsistence on vegetarian diets.

Lusk (6) points out that Loewy and Zuntz, who experienced drastic reduction of diet during the war, both had a greater decrease in metabolism than in weight. Benedict's young men on a semi-starvation diet lost less than 10 per cent of their weight, but at the same time showed a 16–27 per cent lowering of their basal metabolism.

Recent information on the dietary habits of college women alone is limited. Some of the studies available are summarized in Table I, along with part of the data presented in this paper.

TABLE I  
FOOD CONSUMPTION OF COLLEGE WOMEN

Group	Date	No. Observed	Method	Calories	Protein
Chicago University*	1894		Inventory	3277	gm. 108
Wesleyan University*	1894	34	Inventory—10 days	2544	84
North Dakota Agr. College*	1896		Inventory	2589	64
Lake Erie College*	1900		Inventory	2592	68
Vassar*	1917	115	Inventory—14 days	2698	99
Univ. of Chicago** (underwt.)	1922	18	Individual—computed	1830	57
Ames†	1928	8	Individual—computed. 27 days	2342	65
Wang, Chicago‡	1929	6	Individual—analyzed 14 days	1952	67
Oklahoma University§	1930	20	Individual—computed	1820	62
This Study	1930	17	Individual—analyzed	1990	56

\* Cited by—MacLeod and Griggs. *Jour. Home Econ.*, 1918, 10, 97.

\*\* Blunt and Bauer. *Jour. Home Econ.*, 1922, 14, 226.

† Searle and Arnold. *Jour. Home Econ.*, 1928, 20, 84.

‡ Wang, et al. *This Journal*, 1930, 3, 79.

§ Burton, *Okla. Acad. Sci.*, 1931 *Proceedings*.

It will be observed from the table that either the methods used by earlier workers were grossly in error, or else the present tendency is strongly in the direction of habitually lower food consumption among college women. It is significant that both groups of Oklahoma women had diets very similar in caloric and protein content to those of the underweight group of Blunt and Bauer at Chicago. The Oklahoma women consumed 19 per cent less calories and nearly 10 per cent less protein than did the Ames group.

## METHODS

Eighteen normal college women, mostly upper classmen, served as subjects. Each dietary study consisted of two or more observation periods of the usual self-chosen diet. In ten cases the periods were non-consecutive and only four days in length. In eight cases the periods were consecutive and 7 days each in duration. Seventeen of the women were native Oklahomans.

Food was weighed and sampled as eaten and the samples were pooled into a composite representing one-tenth of the total food intake for the period. The details of these methods have been previously described (Coons, 2).

For determination of total calories the oxycalorimeter was employed. For total nitrogen the Kjeldahl procedure was used, for calcium the McCrudden method with hydrogen ion controlled, and for phosphorus the gravimetric method of Neumann.

Basal metabolism tests were given preceding the dietary study. The technic and apparatus were the same as those used in previous studies here.

## RESULTS AND DISCUSSION

The relation between basal metabolism and caloric intake is shown in Table II. The cases have been arranged in descending order of body weight. The underweight individuals predominate in number. Somewhat exaggerated figures are obtained when the caloric intake is compared to the actual basal metabolism because the latter is low and depressed varying degrees in different subjects. Blunt and Bauer's underweight girls averaged only 519 calories in excess of their basal heat production, which, however, was normal. The caloric intake of the women observed in this study averaged 67 per cent in excess of the actual basal metabolism, but only 50 per cent, 635 calories, in excess of the Harris-Benedict prediction.

The calories per kilo. on a basis of "ideal" weight afford a more common ground for comparison. Thus the intake ranged from 29 to 40 calories per kilo. with an average of 35, whereas on the basis of actual weight the average is 38. Blunt's underweight group averaged 38 calories per kilo., while Wang's women on the self-chosen diets averaged only 35 calories per kilo. of actual weight. With respect to calories, then, these Oklahoma women were receiving diets barely within the lower levels of adequacy.

The protein, however, was less adequate in quantity as well as quality, Table III. On an average they consumed 1.1 grams per kilo. of actual weight, or slightly less than 1.0 grams on a basis of ideal weight. The protein furnished 11 per cent of the total calories. The lowest protein in-

TABLE II  
BASAL METABOLISM IN RELATION TO CALORIC INTAKE

Subject	Ht.	Wt.	Weight deviation	Basal Metabolism		Daily Intake—Calories		
				Deviation DuBois	Cal. per 24 hrs.	Total	Excess of basal	Per kilo.
	cm.	kg.	per cent	per cent				
1	149	68.5	+32	— 4	1388	1874	486	27
2	168	72.6	+22	—13	1407	2214	807	30
3	162	61.2	+ 7	—24	1112	2038	926	33
4	170	58.0	— 6	—17	1230	2182	952	37
5	170	59.8	— 7	—16	1243	2019	776	34
6	157	49.4	— 8	—18	1075	1998	923	40
7	159	50.0	— 9	— 4	1254	1747	493	35
8	160	49.4	—11	—16	1108	1969	861	40
9	168	51.0	—13	—13	1248	1989	741	39
10	155	46.3	—13	0	1267	2132	865	46
11	171	51.5	—14	— 7	1277	1814	537	35
12	163	48.5	—16	— 9	1215	1764	549	36
13	167	49.9	—22	—15	1151	2158	1007	43
14	162	41.0	—24	—10	1189	1781	592	43
15	154	38.0	—26	— 8	1050	2075	1025	54
16	157	42.2	—27	—18	996	2009	1013	48
17	160	46.0	—27	—14	1079	2066	987	45
Av.	162	51.9	— 9	—12	1193	1990	796	38

take tended to be associated with the greatest degree of underweight, indicating that low protein consumption may be a causal factor in the under-nutrition of these women.

Subjects with high basal metabolism did not seem to be living on diets any higher in protein or calories than those with lowest rates. The half of the group with the lowest rates had an average intake of 39.4 calories and 1.12 grams of protein per kilo. of actual weight, the half with highest rates, 38.1 calories and 1.02 grams of protein per kilo.

However, the group consists of too few widely different individuals to permit a conclusion on this point. Since our observation of large numbers in the basal metabolism studies has shown a relation between weight status and the rate of metabolism, and since the underweight women tend to have less adequate diets, perhaps a study of the diets of larger numbers would reveal a closer relation between basal metabolism and habitual food consumption. On the other hand, it is believed from the low basal metabolic rates and from these diet studies, that many "average" weight women in our groups were suffering from a subnormal nutrition which

TABLE III  
DAILY INTAKE OF PROTEIN, CALCIUM, AND PHOSPHORUS

Subject	Protein				Calcium	Phosphorus
	Total	Calories from protein	Per kilo. actual wt.	Per kilo. ideal wt.		
	gm.	per cent	gm.	gm.	gm.	gm.
1	50	11	0.7	0.9	0.77	0.92
2	71	13	1.0	1.2	1.06	1.17
3	58	11	0.9	1.0	1.23	1.27
4	61	11	1.0	1.0	0.76	0.97
5	63	12	1.0	1.0	1.32	1.31
6	59	12	1.2	1.1	1.02	1.13
7	49	11	1.0	0.9	0.73	0.98
8	63	13	1.3	1.1	0.97	1.11
9	59	12	1.1	1.0	0.70	1.26
10	63	12	1.4	1.2	1.16	1.35
11	41	9	0.8	0.7	0.52	0.87
12	44	10	0.9	0.7	0.49	0.82
13	56	10	1.1	0.9	0.96	1.61
14	51	11	1.2	0.9	0.62	1.02
15	50	9	1.3	0.9	0.88	1.34
16	49	10	1.1	0.9	1.12	1.39
17	71	14	1.5	1.2	1.55	1.71
Av.	56	11	1.1	0.97	0.93	1.19

was not manifest in extreme weight variations, but, like that in Benedict's diet squads, was sufficient to depress the basal metabolism sharply. If so, the gap of differences in the food consumption of the under- and average weight women might be actually less than one would expect to find.

The most underweight subjects tended to have the lowest mineral intake, also Table III. Ten of the 17 subjects had less than one gram of calcium daily, three had only about one-half gram, and the average for all was also less than one gram daily. The phosphorus intake was low, nearly one-third of the group receiving less than one gram daily. The data for calcium and phosphorus are presented because, in connection with nitrogen, they describe the dietary more accurately. A diet low in both calcium and phosphorus cannot contain appreciable quantities of milk and eggs. If low in nitrogen, also, meat is probably lacking. In other words, the quality of the protein consumed is even more deficient than the quantity. The diet lists show that meat and eggs were chosen seldom, milk by only a few (cases 3, 5, 10, 16, and 17).

Aside from the fact that the diets on the whole possess a low degree

of adequacy, it is doubtful if dietary studies of even one week's duration, except when made under actual home conditions, can depict the food habits of 15 or more years past. Boarding house or club diets have been observed in most of these cases, but past home diets have had the effect on basal metabolism. For example, there was cause to believe that the diets as measured and analyzed for Cases 3 and 10 were considerably better than those commonly received by these subjects. Wakeman and Hansen (11) have shown clearly the need for considering food habits that are of long standing.

The authors wish to call attention, on the other hand, to the diet of Case 11 in this series. It represents the house diet of a sorority, and among this group in the course of a year there was discovered one case of pellagra, two cases of tuberculosis, and a number of cases of marked undernutrition. Girls content to live on such a diet doubtless were unaccustomed to home diets which differed greatly.

Deficiencies of the above diets seem more apparent when compared to those of three other women given in Table IV. Case 18, a northern woman

TABLE IV  
CALORIC REQUIREMENTS FOR MAINTAINING WEIGHT\*

Case	Weight		B. M. R.		Calories				Protein		
	Kg.	Dev.	Dev. DuBois	Cal. per 24 hrs.	Total	per kilo	Excess of basal		Total	per kilo.	Cal. from protein
		per cent	per cent					percent	gm.	gm.	per cent
18	53.1	-10	- 8.5	1282	3080	58	1798	140	77	1.4	10.0
19	58.9	- 7	-15.9	1243	2565	44	1322	106	74	1.3	11.5
20	57.6	0	-18.1	1141	1953	34	812	71	59	1.0	12.1

\* The authors are indebted to Dr. Ruth Reder for a part of the data used in this table.

who had been in Oklahoma only 3 months, was 13 pounds underweight at the time but had been and continued gaining at the rate of about one pound a week. Unfortunately, a basal metabolism test was not secured until some 8 months after the diet study. The rate was then -8.5 (DuBois) and the subject was retaining her added weight but was not gaining more.

For Cases 19 and 20 the data represent a constant diet which had been followed, with an intermission of one week, over a period of 42 days. In the early part of the study slight adjustment in calories and protein had been made until the subjects maintained their constant weight. The women continued their laboratory duties, which differed somewhat for

the two subjects but were routinely the same. All social activity was curtailed, and rest at night was constant and satisfactory in amount. In these two respects their activity differed from that of the average college woman.

The weight of Case 19 has varied only slightly over a period of 10 years, but Case 20 ordinarily finds difficulty in preventing obesity. The basal metabolism of the two showed practically the same deviation from the DuBois predictions. Case 19 represented in height and weight more nearly the average Oklahoma college woman. She was the same subject as Case 5 in the first series.

The facts from this part of the study indicate that 2000 to 2500 calories daily, or at least 1000 calories in excess of basal metabolism, is a desirable intake for average women of this age and activity. However, more will be needed if weight is to be added or the basal metabolic rate is to be raised.

### Summary

There is evidence that the habitual food consumption of present day college women is lower than it was a generation ago, and is lower among Oklahoma women than among those reported for other sections of the United States.

The protein of the diets analyzed was more deficient than was the calorie content.

The probable relation of these sub-adequate diets to the low basal metabolic rates, previously recorded for the college women of Oklahoma, is discussed, with the tentative conclusion that prolonged undernutrition is one factor in many of the cases of lower metabolism.

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Addendum—The excellent paper by Hetler, R. A., *This Journal*, 1932, 5, 69, has appeared since our manuscript went to press.







# THE EFFECT OF FEEDING IRRADIATED ERGOSTEROL TO COWS ON THE VITAMIN D CONTENT OF MILK†\*

By

W. E. KRAUSS, R. M. BETHKE, AND C. F. MONROE

*(From the Departments of Dairy Industry and Animal Industry,  
Ohio Agricultural Experiment Station, Wooster)*

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THAT milk produced under ordinary feeding conditions is a poor source of vitamin D has been demonstrated, directly or indirectly, by numerous investigators. In our own laboratory this fact became impressed upon us while comparing the vitamin D potency of milk from two groups of cows fed widely different rations. In this work at least 23 cc. of milk were required daily for practically normal calcification in rats fed a rickets-producing ration. On this basis the prevalent incidence of rickets in children could be readily understood, and the problem of producing milk containing a sufficient amount of vitamin D to have therapeutic value immediately suggested itself. Since milk is the principal food of infants, it would seem logical to attempt to have such milk as potent as possible in the antirachitic factor. Our ideal has been to devise a method of producing milk which, when taken in the usual quantities, would provide a sufficient amount of all the necessary nutrients to meet the infant's requirements. This report covers the work done on increasing the vitamin D content of cow's milk through the feeding of irradiated ergosterol.

Various methods have been used to increase the vitamin D content of cow's milk. Different methods of feeding the cow have been tried, assuming, as indicated by the work of McCollum, Simmonds, Becker, and Shipley (1) with rats, that it was possible for vitamin D to enter the blood stream and pass into the milk. On the whole, the use of natural feeds for increasing the vitamin D content of milk has been quite unsuccessful and the results obtained are conflicting. It is generally agreed that milk from pasture-fed cows is richer in vitamin D than milk from stall-fed cows (2, 3, 4); but whether the grass itself or the degree of insolation of the cow is responsible has not been definitely shown. Nor is the increase in vitamin D, resulting from such feeding, of sufficient magnitude to be of great significance.

Irradiation of the cow has been tried in an attempt to increase the vita-

† A brief report of this investigation was presented before the American Society of Biological Chemists at Montreal, Canada, April, 1931.

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min D content of milk, but the results of this type of experiment are very conflicting. Gowen, Murray, Gooch, and Ames (5) succeeded in curing rickets in chicks by feeding milk from cows that had been exposed daily for 15 to 30 minutes to ultra-violet light; while chicks fed milk from control cows became progressively more rachitic. Falkenheim, Völtz, and Kirsh (6) showed that milk from cows exposed to light from a quartz-mercury vapor lamp had definite antirachitic power; whereas that from cows not exposed had practically none. On the other hand, Steenbock, Hart, Rising, Hoppert, and Basherov (7) were unable to increase appreciably the antirachitic potency of milk by irradiation of the cow.

Direct irradiation of milk or butterfat has been shown by numerous investigators to increase the vitamin D content to such an extent that milk or fat so treated has definite therapeutic value in the treatment of rickets (8, 9, 10, 11, 12, 13, 14, 15, 16). The objections first raised to this method of treating milk—namely, that vitamin A was destroyed and a bad flavor was imparted to the product—are now being overcome by improved methods for carrying out the irradiation process (17).

Still another line of attack followed in this problem consisted of feeding vitamin D concentrates. As early as 1924 Lesne and Vagliano (18) had succeeded in increasing the vitamin D content of milk by feeding cod liver oil to cows. This was later confirmed by Golding, Soames, and Zilva (19) who in turn confirmed the previous observation of Drummond, Channon, Coward, Golding, Mackintosh, and Zilva (20) that relatively high doses of cod liver oil depressed the fat percentage of cow's milk. A similar observation was made by the Wisconsin workers (21). A satisfactory method for increasing the vitamin D content of milk without affecting the fat content consists of feeding cows irradiated yeast, as shown by Wachdel (22) and by Steenbock, Hart, Hanning, and Humphrey (23). Thomas and MacLeod (24) have shown that the feeding of irradiated yeast or irradiated ergosterol to cows increased the vitamin D content of milk, and Hess, Lewis, MacLeod, and Thomas (25) have shown that these milks have therapeutic value in the treatment of rickets in infants.

The discovery of irradiated ergosterol as the most potent known source of vitamin D, together with a knowledge of the experience of others in attempting to increase the antirachitic potency of milk, led to the adoption of this product as the source of vitamin D in the work here reported.

#### EXPERIMENTAL

As a preliminary trial, during different periods, a Jersey cow was fed, mixed with her grain allowance, 5, 10, 25, and 100 mg. of irradiated ergos-

terol in corn oil daily. In this preliminary work the ergosterol used was not re-assayed in our own laboratories, but the evaluation given by the manufacturer<sup>1</sup> was used in calculating the number of rat units fed. On this basis, the amounts of ergosterol mentioned above contained 6,500, 13,000, 32,500, and 130,000 Steenbock rat units, respectively. Use of the standard prophylactic (bone-ash) and curative (line-test) procedures of assaying for vitamin D revealed that the antirachitic value of the butterfat produced by this cow was not appreciably increased when 5 mg. or 10 mg. of ergosterol were fed. However, at the 25 mg. level there was a slight indication that the antirachitic potency of the butterfat had been increased; whereas at the 100 mg. level the resulting increase in vitamin D was very marked.

These observations warranted further work with more animals. Consequently, three Holstein cows, in approximately the same stage of lactation and producing about the same amount of milk, were selected (Table I). Because one of these cows had to be removed from the herd shortly after the beginning of the experiment, the data contained in this paper are based upon the fat production of two cows.

TABLE I  
DATA ON COWS WHEN STARTED ON EXPERIMENT

No. Breed	Date of last calving	Stage of lactation	State of gestation	Daily milk production
236 Holstein	Jan. 18, 1930	73rd day	Not pregnant	35 lb.
285 Holstein	Jan. 4, 1930	87th day	Not pregnant	35 lb.

The cows were kept under winter feeding conditions throughout, except for a short period each day when they were allowed in a courtyard free from vegetation in order to obtain water and exercise. The daily ration consisted of alfalfa hay of fair quality, corn silage, corn, oats, bran, and linseed oil meal. At the beginning of the trial sufficient hay was set aside to last throughout the experiment.

In order to measure the full effect of any feeding program, it was assumed that a period of at least three weeks must elapse; therefore, the periods indicated in Table II are of approximately three weeks' duration.

Periods 1, 4, and 7 were controls, during which time 50 cc. of corn oil<sup>2</sup> were fed in order to equalize the additional fat intake necessitated during the ergosterol periods. In periods 2 and 3, ergosterol furnished by the

<sup>1</sup> Standard Brands Incorporated.

<sup>2</sup> Mazola.

TABLE II  
EXPERIMENTAL FEEDING PLAN

Period No.	Feeding period	Supplement fed daily	Rat units of Vitamin D fed Daily*
1	April 1-24 (24 days)	50 cc. corn oil	—
2	Apr. 25-May 15 (21 days)	25 cc. ergosterol solution 25 cc. corn oil	7,500
3	May 16-June 5 (21 days)	50 cc. ergosterol solution	15,000
4	June 6-July 10 (35 days)	50 cc. corn oil	—
5	July 11-July 31 (21 days)	10 cc. ergosterol solution 40 cc. corn oil	100,000
6	Aug. 1-Aug. 26 (26 days)	20 cc. ergosterol solution 30 cc. corn oil	200,000
7	Aug. 27-Sept. 26 (31 days)	50 cc. corn oil	—

\* Each sample of ergosterol was re-assayed for vitamin D in our own laboratories and the potency expressed in terms of the Steenbock rat unit. This system of expressing vitamin D potency is used throughout the paper.

Acetol Products Corporation<sup>3</sup> was used; in periods 5 and 6 the ergosterol was a product furnished by Standard Brands, Inc.<sup>3</sup> The pure corn oil or the ergosterol-corn oil mixture was mixed with the daily grain allowance of each cow.

During the last five days of each period the total amount of milk produced by both cows was collected and combined. This was then separated and the cream churned. The resulting butter was then rendered into pure fat by melting at the lowest possible temperature, washing with hot water, and filtering through funnels enclosed in a cabinet warmed with carbon filament electric light bulbs. The resulting fat was then placed in Mason jars and stored in a cooler slightly above freezing temperature until used.

A small portion of each sample of fat was taken from the original samples daily and melted in a beaker over a low flame. The required amount

<sup>3</sup> The authors are indebted to the Acetol Products Corporation, New Brunswick, N. J. and Standard Brands, Inc., New York City, for furnishing the ergosterol solutions.

was dropped out of a calibrated eye dropper into a small glass dish which was then placed in the designated cage.

The rats were of our own breeding. In the curative (line-test) procedure they were weaned when they weighed approximately 50 to 55 grams (24 to 26 days of age) and placed by litters in wire cages with screen bottoms in which they had free access to Steenbock and Black's rickets-producing ration (24).<sup>4</sup> At the end of three weeks the rats were examined for rickets and, if suitable, were transferred to individual cages and distributed in such a way that a representative of each litter was in each group, as far as possible. The criteria used for determining the incidence of rickets were enlargement of the wrists and depression of the thoracic region. Gain in weight proved to be a reliable index of the development of rickets on this ration. Animals gaining less than 15 to 20 grams usually did not exhibit the external symptoms of rickets and were discarded. During the butterfat feeding period, careful record of feed consumption was kept.

At the end of 10 days the rats were etherized and then killed by severing the jugulars and carotids. The blood from each rat was analyzed for inorganic phosphorus but, since the results obtained were of no particular significance and for the sake of brevity, they have been omitted. The radii and ulnae were removed, preserved in 10 per cent formalin, and subsequently examined for degree of calcification by the silver-nitrate method. The results, in part, are recorded in Table III.

TABLE III  
THE CRITICAL AMOUNT OF BUTTERFAT REQUIRED, DAILY, TO PRODUCE DEFINITE  
EVIDENCE OF HEALING IN THE RAT

Fat sample	Units of Vitamin D fed to cows daily	No. of rats	Critical daily level of butterfat	Rat units per gram of butterfat
			mg.	
1	0	10	600	0.17
2	7,500	5	350	0.29
3	15,000	4	200	0.50
4	0	5	120	0.83
5	100,000	5	60	1.67
6	200,000	5	40	2.50
7	0	4	250	0.40

While only data for the critical level of butterfat—that is, the minimum at which definite healing occurred—are presented, it should be pointed

<sup>4</sup> 76 yellow corn, 20 wheat gluten, 3 calcium carbonate, 1 salt.

out that many different levels, above and below the one indicated in the table, were fed in order to establish these critical values. From the data recorded in Table III it is obvious that as the vitamin D intake of the cows increased, the antirachitic potency of the butterfat increased correspondingly, from 0.17 Steenbock rat units per gram during the control period (No. 1) to 2.5 units per gram where the vitamin D intake, in the form of ergosterol, amounted to 200,000 rat units daily (period 6). One discrepancy appears to exist in the data. During period 4 no ergosterol was fed; yet the vitamin D potency of the butterfat produced at the end of this period was greater than that of the fat produced at the end of period 3 when 15,000 rat units of vitamin D were fed daily. That this was not due to an error in assaying is indicated by the prophylactic trials (Table IV). Here, better calcification was secured on 200 mg. of butterfat 4 than on 200 mg. of butterfat 3. The explanation that there was a storage of the vitamin is obviated by the values obtained for butterfat 7 (Tables III and IV). No satisfactory explanation of this has suggested itself.

In the prophylactic procedure the rats were placed in individual cages at weaning time (24 days) and allowed free access to the Steenbock and Black diet. A representative of each litter was placed in each group, including the negative and positive controls, as far as possible. The negative controls received the basal ration only; the positive controls received the basal ration plus 2 per cent of a standardized cod liver oil. The butterfat allowances were fed separately each day for five weeks. At the end of that time the rats were chloroformed and the femurs removed. After the removal of all tissue the bones were dried, extracted for 36 hours in 95 per cent alcohol and then in ether for 24 hours. The extracted bones were then dried and ashed. The ash results, given in Table IV, are expressed on a fat- and moisture-free basis.

The data in Table IV show that better calcification was obtained prophylactically with 40 mg. of butterfat 6 than with 600 mg. of butterfat 1, or, in other words, when 200,000 units of vitamin D were fed, the antirachitic potency of the resulting butterfat was at least 15 times as great as that obtained from feeding the ordinary dairy ration (period 1). These results agree closely with those obtained by the line-test method.

In order to obtain further information with another species as to the comparative calcifying values of butterfat samples 4, 5, and 6, the chick was chosen as the experimental animal. For this purpose 10 lots of 15 day-old White Leghorn chicks each, of the same parentage, were started on experiment. All the lots were confined indoors in brooders provided with screen bottoms and were fed a leg weakness-producing ration of yellow

corn 46 parts, wheat 20, wheat bran 5, soybean meal 20, dried buttermilk 5, steamed bone meal 3, salt 1, and corn oil<sup>6</sup> 5. The different percentages of butterfats and cod liver oil were incorporated in the above ration by replacing an equivalent amount of corn oil. The cod liver oil, like the butter-

TABLE IV  
THE EFFECT OF DIFFERENT SAMPLES OF BUTTERFAT  
ON CALCIFICATION IN RATS

Fat samples	Units of vitamin D fed to cows daily	Amount of butterfat fed daily	No. of rats	Average gain in weight	Average ash in femurs
		mg.		gm.	Per cent
1	0	400	5	28.6	37.72 ± 1.21
		600	5	33.2	43.71 ± 1.23
2	7,500	200	4	28.8	37.85 ± 1.46
		400	5	29.0	42.67 ± 0.29
3	15,000	100	5	28.6	32.59 ± 1.69
		200	4	36.0	41.43 ± 0.86
4	0	180	5	21.2	48.32 ± 0.41
		200	4	27.0	48.54 ± 0.32
		400	5	23.8	51.60 ± 0.48
5	100,000	80	5	41.4	50.11 ± 0.73
		100	4	29.8	51.94 ± 0.45
		200	5	32.6	54.17 ± 0.41
6	200,000	40	4	32.8	49.27 ± 0.89
		50	5	27.6	47.88 ± 0.61
		100	5	28.4	50.09 ± 0.32
		120	4	44.5	49.36 ± 0.62
7	0	100	5	41.2	38.12 ± 1.04
		200	5	46.8	44.42 ± 1.04
Negative controls			17	25.8	30.23 ± 0.57
Positive controls (2 per cent C.L.O.)			16	31.3	50.55 ± 0.41

fats, had been previously assayed for vitamin D. All the chicks were weighed individually each week. At the end of 6 weeks 10 representative birds from each lot were killed for blood and bone analyses. Calcium (27) and inorganic phosphorus (28) determinations were made on the pooled

<sup>6</sup> Mazola.



serum of each group. Ash determinations were made on the tibiae after thorough extraction of the dried, crushed bones with alcohol and ether in Soxhlet extractors. The data obtained are presented in Table V.

Although the evidence obtained with the chicks is not so striking as that obtained with the rats, it, nevertheless, substantiates the rat work inasmuch as the ash content of the bones of the chicks in lot 6 (5 per cent butterfat 6) is significantly greater than that of the bones of the chicks in lot 2

TABLE V  
EFFECT OF DIFFERENT SAMPLES OF BUTTERFAT ON CALCIFICATION IN CHICKS

Lot No.	Per cent of fat substituted for corn oil in basal ration	Units of vitamin D per 100 gm. ration	Average weight at 6 weeks	Blood Analysis		Average ash in tibiae
				Ca. per 100 cc. serum	P. per 100 cc. serum	
1	None	No. 0	gm. 156.2	mg. —	mg. 6.92	Per cent 40.79 ± 0.38
2	5.0 per cent butterfat No. 4	4.2	241.7	8.6	7.15	43.59 ± 0.36
3	2.0 " " " No. 5	3.3	214.7	8.1	6.83	42.89 ± 0.46
4	5.0 " " " No. 5	8.3	213.2	8.5	6.35	44.34 ± 0.43
5	2.0 " " " No. 6	5.0	194.1	7.4	7.15	42.47 ± 0.29
6	5.0 " " " No. 6	12.5	228.2	8.8	6.88	45.76 ± 0.45
7	0.1 per cent cod liver oil	3.3	305.9	9.9	7.15	47.53 ± 0.18
8	0.2 " " " " "	6.7	292.9	11.1	7.43	50.08 ± 0.14
9	0.3 " " " " "	10.0	310.4	10.0	7.35	49.60 ± 0.45
10	0.5 " " " " "	16.7	298.4	11.3	6.85	50.79 ± 0.31

(5 per cent butterfat 4). The data also indicate that cod liver oil was more efficient than these butterfats as a source of vitamin D for chicks, since better calcification was obtained from fewer units of vitamin D in the form of cod liver oil than in the form of butterfat. On a rat unit basis it required more than four times as many vitamin D units in butterfat (Lot 6) as in cod liver oil (Lot 7) to bring about the same degree of calcification. These observations are of interest in view of the findings of Mussehl and Ackerson (29) and of Massengale and Nussmeier (30) that the chick requires

several times more rat units of vitamin D in the form of irradiated ergosterol than in the form of cod liver oil.

It has been pointed out by Hart, Steenbock, Teut, and Humphrey (31) and by Hart, Steenbock, Kline, and Humphrey (32) that the vitamin D in cod liver oil and in irradiated yeast is poorly absorbed from the intestinal tract of cows. That vitamin D in the form of irradiated ergosterol dissolved in corn oil is also poorly absorbed, or else stored in some organs other than those concerned with milk secretion, is demonstrated in our work (Table VI).

TABLE VI  
RELATION BETWEEN AMOUNT OF VITAMIN D FED AND VITAMIN D CONTENT OF BUTTERFAT

Sample	Weight	Rat units per gram	Total rat units in sample	Rat units fed to cows
	gm.			
1	4,319.2	0.17	734.3	—
2	5,616.8	0.29	1,628.9	75,000
3	5,226.6	0.50	2,613.3	150,000
4	3,665.9	0.83	3,042.7	—
5	4,028.9	1.67	6,728.3	1,000,000
6	4,001.6	2.50	10,004.0	2,400,000
7	2,459.1	0.40	983.6	—

## DISCUSSION

There is now sufficient evidence to show unmistakably that the vitamin D content of milk can be materially increased. The question as to which method, direct irradiation of the milk, the feeding of irradiated yeast, the feeding of irradiated ergosterol, or of some other concentrated source of vitamin D, is most practical, will depend upon future developments in the field of ultra-violet illumination and improved methods of manufacturing vitamin D concentrates in order to reduce their cost. The efficiency of the source of vitamin D used must also be determined. Already, there are indications that cows use the vitamin D in irradiated yeast much more efficiently than that in irradiated ergosterol (25).

It would seem that the natural method for increasing the vitamin D content of milk would be through feeding the cow. This would eliminate special handling of the milk after it was produced, as is necessary when milk is irradiated. However, it would be very difficult to follow closely the potency of a milk supply whose vitamin D content was dependent upon feeding operations carried on over scattered areas. Until rapid chemical

methods for vitamin assays have been perfected, such control could not be expected and it is perhaps only for special, carefully supervised and scientifically handled herds producing special kinds of milk that the practice may be at all warranted.

Another question that arises concerns the effect upon the physical well-being and performance of the cows of feeding irradiated ergosterol. Work with other species of animals has shown that excessive doses of irradiated ergosterol are toxic. Pathological studies of the internal organs of cows that were fed ergosterol for long periods of time are now in progress. It can be said at this time that, so far as external symptoms, production, and reproduction are concerned, there is no indication that the feeding of 200,000 rat units of irradiated ergosterol in corn oil daily over a period of almost a year was detrimental.

Although in a problem such as this certain fundamental truths can be ascertained with small animals like rats and chicks, the real criterion as to the value of these facts must be based upon work with the species to be benefited. Accordingly, arrangements were made with the Babies and Childrens Hospital at Cleveland, Ohio, to feed milk produced by ergosterol-fed cows in the Ohio Experiment Station herd to rachitic babies. The results of this work are presented in the following paper.

#### SUMMARY

Two Holstein cows in the same stage of lactation, kept under winter feeding conditions, and consuming a good dairy ration, were fed various amounts of irradiated ergosterol dissolved in corn oil, over three-week periods. The vitamin D content of representative samples of butterfat collected during each period was determined biologically, by both the curative (line-test) and prophylactic (bone-ash) procedures, and compared with the vitamin D content of butterfat from the same cows when an equal volume of corn oil was fed. The antirachitic potency of the butterfat increased as the number of rat units of vitamin D fed increased—from 0.17 Steenbock rat units per gram during the control period to 2.5 units per gram when 200,000 rat units of the antirachitic factor were fed. This relationship was confirmed by bone-ash values obtained in prophylactic trials.

Evidence is presented showing that vitamin D in cod liver oil is more efficient for calcification in chicks than that contained in butterfat from cows fed irradiated ergosterol.

The practicability of feeding cows irradiated ergosterol so as to produce milk rich in vitamin D is discussed.

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# THE TREATMENT OF RACHITIC INFANTS WITH MILK PRODUCED BY COWS FED IRRADIATED ERGOSTEROL

By

HENRY J. GERSTENBERGER AND ARTHUR J. HORESH

*(From the Babies and Childrens Hospital and the Department of Pediatrics of Western Reserve University School of Medicine, Cleveland)*

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THE following report presents observations made by us at the Babies and Childrens Hospital on two rachitic infants who were fed milk produced by cows given 100 mg. of irradiated ergosterol daily, having an antirachitic value of 200,000 rat units, as outlined in the preceding article (1). The infants were fed this milk after a preliminary period of observation of four weeks, during which the character and the degree of the rickets were established. The milk was from cows at the Ohio Agricultural Experiment Station, Wooster, Ohio.

Both infants received daily 500 cc. of this whole milk together with 500 cc. of ordinary skim milk to which were added 5 cc. of lactic acid and enough carbohydrate to meet the caloric requirements of the infants. In addition 15 cc. of orange juice were administered daily. A mixture of half whole and half skim milk is, in our opinion, a much safer food than whole cow's milk from the standpoint of avoiding diarrhea over a long period, particularly when infections develop. We also are convinced that it is wiser to use a pint of milk rather than a quart as the antirachitic unit for cow's milk, inasmuch as most infants during the greater part of their first year will receive within twenty-four hours no more fat than is contained in a pint of milk.

Unfortunately, the parents of the infants insisted on taking them home before complete healing of the bones had occurred. The patients, however, did remain in the hospital long enough to enable us to know that the milk contained definite antirachitic powers and to assure us that the rickets would have healed completely had the time been extended beyond the period of ten weeks for the one infant and eleven and a half weeks for the other. These time periods are adequate to bring the blood serum calcium and phosphorus figures to normal levels and the roentgenograms of the radius and ulna to present a state of complete or nearly complete calcification when rachitic infants are exposed once per week to an erythema-

producing dose of a quartz mercury vapor arc lamp (2) or of the Sunlight (Type S-1) lamp (3) or when they are given 5 cc. of cod liver oil a day (Fig. 3). In certain food mixtures<sup>1</sup> even smaller quantities of cod liver oil have been found to have an antirachitic potency of this degree.

It is evident that the unquestionable healing which was produced in these infants as the result of the ingestion of the 500 cc. of the "Wooster

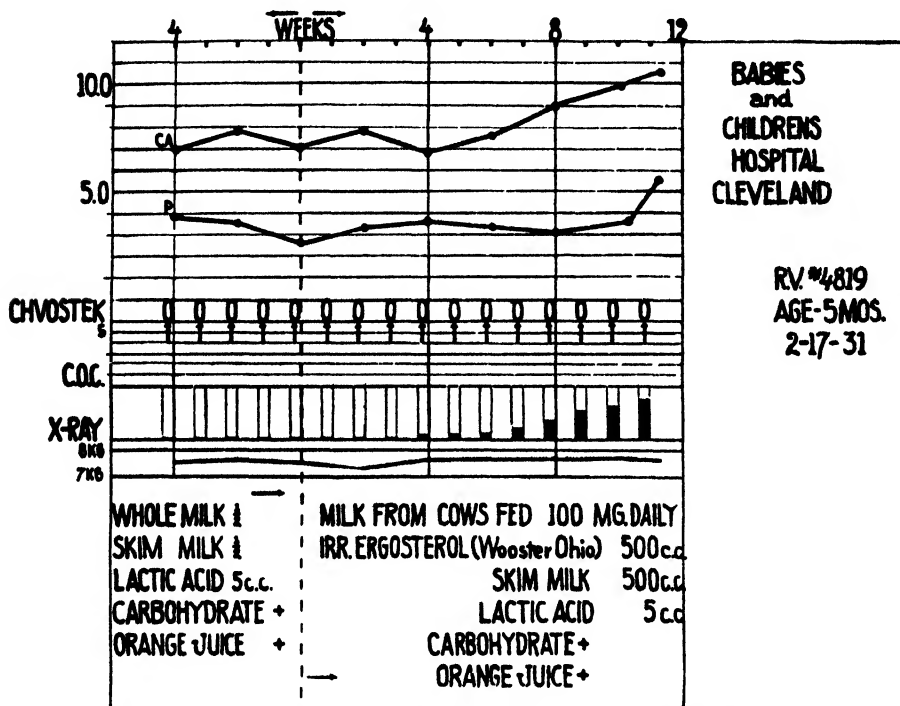


FIG. 1. Progress in patient R. V. In Figures 1, 2 and 3 Chvostek 0 means negative Chvostek; Chvostek +, one plus Chvostek. The black triangles indicate the presence of the Erb phenomenon (C.O.C., cathodal opening contraction), and the arrow indicates that the C.O.C. requires more than 5 milliamperes. The columns consisting of three lines represent moderate rickets; the columns consisting of two lines represent marked rickets. The black areas indicate the presence of healing, and the height of the black areas the extent of the healing. The vertical dotted line indicates the end of the four-week observation period and the beginning of treatment.

milk," relatively speaking, was slow. On the basis of our practical experience we would judge it to be the equivalent of somewhat less than a daily dose of one-half teaspoonful of a cod liver oil giving in a dose of 5 mg. per day adequate antirachitic protection to rats.

The graphic charts (Figs. 1, 2 and 3) present clearly the course of events.

<sup>1</sup> S. M. A. and Protein S. M. A.

It will be noted that the first evidence of healing in the roentgenograms for patient R. V. was observed in the fourth week and for patient M. H. in the third week after beginning of treatment. The calcium content of the blood in both patients reached the normal level at ten weeks, while the phosphorus content, on the other hand, only reached the normal level for patient R. V. in the eleventh week and did not reach the normal level in patient

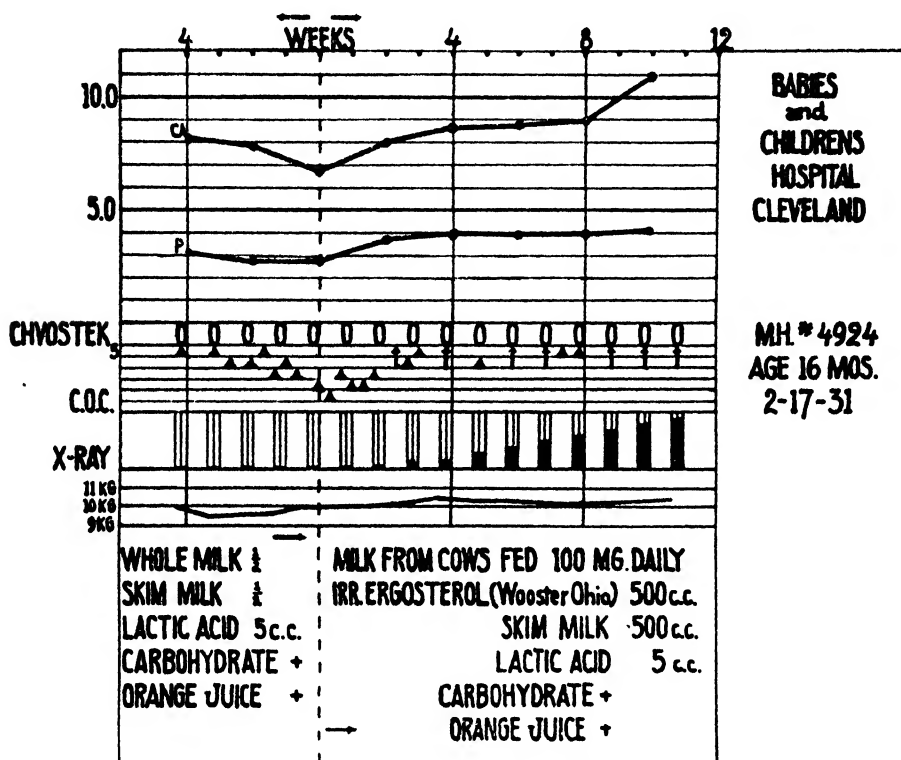


FIG. 2. Progress in patient M. H.

M. H. at the tenth week when she was discharged, it being still at the level of 4.0 mg. In other words, at the end of the treatment periods the bones were not completely healed. Further evidence of the mildness of the anti-rachitic quality of the milk is the fact that the spasmophilic symptoms in the one infant did not completely disappear until the eighth week.

### SUMMARY

1. Two rachitic infants, after a preliminary treatment-free observation period of four weeks to determine the type and the degree of rickets present, were fed 500 cc. of whole milk produced at the Ohio Agricultural Ex-



periment Station, Wooster, by cows fed daily 100 mg. of irradiated ergosterol having an antirachitic value of 200,000 rat units.

The daily food mixture for the infants contained in addition 500 cc. of ordinary skim milk, 5 cc. of lactic acid and a sufficient amount of carbohydrate to meet the caloric requirements of the infants. Fifteen cubic centimeters of orange juice also were administered.

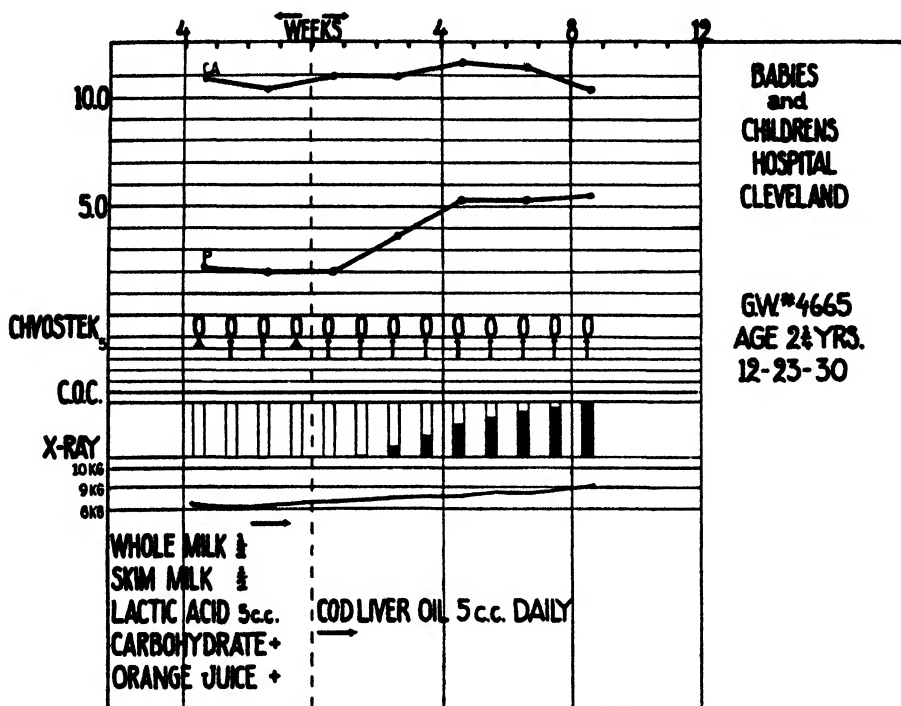


FIG. 3. Progress in patient G. W. receiving 5 cc. of cod liver oil daily. Shown for comparison with Figures 1 and 2.

2. The observations made during the treatment period, which for the one infant lasted ten and for the other eleven weeks, showed conclusively, by the use of weekly roentgenograms and bi-weekly blood serum calcium and phosphorus determinations, that the milk possessed antirachitic powers, but that these were not of a great magnitude. The bones were not completely healed at the end of the treatment periods, the calcium levels rose to normal only at the tenth week and the phosphorus level in the one infant at the eleventh week and in the other infant not yet at the tenth week when she was released from the hospital. Further evidence of the mildness of the antirachitic quality of the milk is the fact that the spasm-

philic symptoms in one infant did not completely disappear until the eighth week.



On the basis of practical experience with the feeding of cod liver oil having a rat protective potency of 5 mg. per day, it is estimated that a pint of this particular milk contained slightly less than the equivalent of one-half teaspoonful of such a cod liver oil.

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# THE EFFECT OF DAIRY MANUFACTURING PROCESSES UPON THE NUTRITIVE VALUE OF MILK

## I. THE APPARENT DIGESTIBILITY OF FRESH WHOLE MILK AND OF EVAPORATED MILK

By

W. B. NEVENS AND D. D. SHAW

*(From the Department of Dairy Husbandry, University of  
Illinois, Urbana)*

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**D**URING a study of the effect of various manufacturing processes upon the nutritive properties of milk, a superiority of evaporated milk over fresh whole (raw) milk as food for young albino rats was demonstrated. It was assumed that differences in digestibility might be one of the factors responsible for the nutritive differences and trials were conducted to determine the correctness of this assumption.

The literature on infant feeding is replete with references to the "ease" of digestibility of evaporated milk, particularly of its protein. The present study, however, deals only with *completeness* of digestibility and has no bearing upon the "ease" or time factor.

Daniels and Loughlin (3) observed a less satisfactory growth in rats fed evaporated milk than in those fed quickly boiled milk or sweetened condensed milk. It was found that the manufacturing processes then in use permitted a settling out of salts from the milk and that when these were incorporated in the feedings the differences in growth were largely overcome.

Magee and Harvey (5) report poorer retention of calcium, phosphorus, and nitrogen in pigs fed pasteurized than in those fed fresh or sour milk.

Morgan (7) studied the biological values of wheat gluten, whole wheat proteins, and casein both raw and after toasting at 200° C. for 45 minutes. Heating lowered the biological values but affected digestibility only a little.

Rettger (9) reports the partial decomposition of milk proteins with liberation of volatile sulfide on heating above 85° C., the pasteurized and condensed milk liberating smaller amounts of sulfide than normal milk.

Washburn and Jones (12) found that young pigs stored the nitrogen of raw milk somewhat better than that of evaporated milk.

Some investigators, on the other hand, have found that the heating or cooking of proteins increases their nutritive value. Bateman (1) reports

that dogs utilized raw egg white to the extent of only 50 to 70 per cent compared with a utilization of 90 per cent for cooked egg white. Little effect was obtained by heating to less than 55° C., while best results were secured at temperatures of 65° to 70° C. or above. Johns and Finks (4) found that heating phaseolin suspended in water and boiled 30 minutes enabled it to produce normal growth, whereas the unheated protein did not support normal growth. In both cases it was supplemented with cystine. Mendel and Fine (6) also found that heating phaseolin increased its value, and Waterman and Johns (13) report detectable increases in digestibility of this protein through cooking. Waterman and Jones (14) secured increased digestibility of the proteins of the Chinese and Georgia velvet beans by coagulating and cooking.

Wallen-Lawrence and Koch (11) compared the digestibility by trypsin *in vitro* of 232 samples of evaporated milk, boiled milk, and raw milk. In every case the digestibility of the heated milk was higher.

Brennemann (2) states that as yet no inferiority of evaporated milk compared with fresh milk has come to light that cannot apparently be neutralized by the use of orange juice and possibly cod liver oil.

Willard and Blunt (15), in three out of four children, observed a higher nitrogen retention when they were given evaporated milk than when they were given pasteurized milk.

#### EXPERIMENTAL METHODS

The feeding and management of the experimental rats during the digestion trials were essentially the same as described in an earlier publication (Nevens and Shaw, 8). Instead of using shavings in the pans below the glass cages, however, the pans were lined with sheets of heavy filter paper 20" by 20" in size, held in place by paper clips. A thin wood strip was employed as a division midway across the pan.

Two brands of commercial evaporated milk designated as Brands "R" and "B" were employed. In addition evaporated milk designated as Brand "D" was prepared in the laboratory. The milk for its preparation was milked directly from a cow into a glass funnel and glass jug. The fresh (raw) milk fed in comparison with all three brands was secured daily from the same cow in the same manner. The milk thus obtained (about one gallon in amount) comprised only one-half to three-fourths of the milk yielded by the cow at that particular milking. The term "whole milk" as used in this paper, therefore, refers to milk secured by this method. Evaporation was carried out in a 12-liter round-bottom glass flask. The flask was placed in a water bath and the temperature of the milk brought

to about 93° C. The temperature was lowered and evaporation to approximately one-half the original volume carried out under reduced pressure at a temperature of 55–60° C. The milk was transferred to sterile 200 cc. Erlenmeyer flasks stoppered with cotton. These were heated in an autoclave with steam pressure of 10 pounds for 15 minutes. All milk supplies were stored in a refrigerator. The fresh milk was sampled daily and a sample was taken from each container of evaporated milk as opened.

The digestion trials were ten days in length. No preliminary periods were necessary in the case of animals Nos. 155–190, since they had been fed continuously in the same manner since weaning.

The use of animals Nos. 155–190 for digestibility studies might naturally draw criticism because of their being in an anemic condition. Some of them died before the experiments were completed. To meet this objection, animals normal in hemoglobin content were selected from our stock colony and paired according to sex and approximate weights. These animals were Nos. 191–228. They were fed stock mixture following each of the digestion trials but allowed milk only for three to five days preceding the next trial.

Two trials of the digestibility of the protein of each of the commercial brands of evaporated milk were conducted with the stock animals. In the second trial, animals which received fresh milk in the first trial were given evaporated milk and vice versa. Another criticism of the feeding of animals Nos. 155–190 (Table I) is that the animals fed whole milk consumed slightly less protein than those fed evaporated milk. In the trials with the stock animals, therefore, animals receiving whole milk were fed two times the volume of milk consumed by their pair mates and 10 per cent in addition. This brought the level of protein intake of the whole-milk animals above that of their pair mates. Water was supplied in separate dishes to animals being fed evaporated milk.

Feces were collected daily. In the studies of protein, the feces were transferred at once to stoppered Kjeldahl flasks containing 50 cc. of C.P. sulfuric acid. When it was evident that some of the feces had been contaminated by urine, they were transferred to a sheet of clean filter paper and washed before collection by a stream of hot, acidified, nitrogen-free water from a wash bottle.

For determinations of the other nutrients, the feces were transferred to glass jars which were kept in a refrigerator. The same samples of feces were used for dry-matter and ether-extract determinations, but separate collections were made for sugar determinations. The methods of analysis prescribed by the Association of Official Agricultural Chemists were followed

in the analysis of milk and feces. Only qualitative tests for sugar were made. Both Benedict's and Fehling's methods were employed.

Calculations of digestibility were made by assuming that the differences between the amounts of the nutrients ingested and the amounts of the same nutrients appearing in the feces represent the amounts digested. On account of the presence of small quantities of products of endogenous origin in the feces, coefficients calculated in this manner are in reality coefficients of "apparent" digestibility. For the sake of simplicity, however, the term "apparent" has been omitted in the body of this paper.

### DISCUSSION OF RESULTS

Fresh whole milk proved superior to both commercially evaporated and laboratory-evaporated milk in the digestibility of its protein (Tables I, II). The data are fairly consistent for the different animals and different brands of milk. The mean of the 47 coefficients of digestibility for fresh whole milk is  $92.3 \pm .17$ . The 45 coefficients for the three brands of evaporated milk have a mean of  $88.4 \pm .25$ .

There is quite a high degree of probability that the differences shown by pair mates in the digestibility of protein (Tables I, II) signify an actual difference in the nutritive character of the two kinds of milk. It is calculated by the method of Student (10) that in an experiment with pair mates an average difference as great as that found with animals fed fresh whole milk and Brand "R" evaporated milk (Table I) would be expected to occur by chance but once in 39 trials. The probabilities that average differences in digestion coefficients as large as those found in the other trials were brought about by factors other than chance alone are as follows: For Brand "R", evaporated milk, the probability is 145:1; for Brand "D", 600:1; for Brand "R" (Table II, Period 1), 42:1; for Brand "R" (Table II, Period 2), 303:1; for Brand "B" (Table II, Period 3), 2,700:1; for Brand "B" (Table II, Period 4), more than 10,000:1.

A point deserving of special emphasis is brought out by the reversal trials (Table II). In these two sets of trials every animal, with but a single exception, had a higher coefficient of digestibility of protein when fed fresh whole milk than during the corresponding period when fed evaporated milk. This was true whether the fresh whole-milk feeding preceded or followed the evaporated-milk feeding. These data, therefore, present a strong array of evidence that, under the conditions of this experiment, the digestibility of the protein of fresh whole milk is greater than that of evaporated milk.

The fat of milk was found to be very highly digestible (Table III). Of the

TABLE I  
COMPARATIVE DIGESTIBILITY OF TOTAL PROTEIN OF FRESH WHOLE MILK AND EVAPORATED MILK

Animal No.	Live weight grams	Protein in milk consumed grams	Coefficient of digestibility	Animal No.	Live weight grams	Protein in milk consumed grams	Coefficient of digestibility
Fresh whole milk				Brand R evaporated milk			
155	130	13.9	91.6	156	184	14.3	89.2
157	102	12.1	92.6	158	106	12.3	90.4
159	109	13.1	92.3	160	190	13.5	91.2
Fresh whole milk				Brand B evaporated milk			
163	107	12.8	95.8	164	122	11.1	90.9
165	126	14.3	95.7	166	153	12.5	87.6
167	133	16.7	93.4	168	174	14.6	86.9
171	108	12.2	92.8	172	135	10.4	82.7
Fresh whole milk				Brand D evaporated milk			
173	130	15.7	93.7	174	119	15.1	94.1
175	119	14.4	94.1	176	122	13.9	90.2
177	127	13.7	94.1	178	159	13.2	91.1
179	113	12.7	96.1	180	130	12.3	92.7
181	106	14.7	94.3	182	148	14.2	89.4
183	113	13.3	94.2	184	130	12.8	91.6
185	132	14.2	94.8	186	144	13.7	91.1

Note: In this, as in Tables II and III, records of pair mates are shown on the same line. Thus, No. 155 is the pair mate of No. 156, etc.



TABLE II  
COMPARATIVE DIGESTIBILITY OF TOTAL PROTEIN OF FRESH WHOLE MILK AND EVAPORATED MILK

Animal No.	Live weight grams	Protein in milk consumed grams	Coefficient of digestibility	Animal No.	Live weight grams	Protein in milk consumed grams	Coefficient of digestibility
Period 1. February 20 to March 2, 1931							
Fresh whole milk				Brand R evaporated milk			
191	330	18.6	93.4	192	330	15.7	88.9
193	298	17.8	90.9	194	302	14.5	90.5
195	284	21.1	*	196	265	18.1	91.5
197	143	17.4	92.9	198	155	14.8	88.1
199	150	17.4	92.1	200	119	14.8	88.7
201	195	13.9	92.3	202	215	**	
203	284	16.2	92.7	204	224	**	
Period 2. March 13 to March 23, 1931							
Brand R evaporated milk				Fresh whole milk			
191	345	15.1	87.2	192	337	19.5	92.4
193	327	18.6	89.7	194	320	24.1	88.9
195	302	14.4	87.4	196	285	18.7	91.9
197	200	18.9	85.6	198	220	24.0	90.7
199	225	16.2	85.9	200	182	20.4	90.2
201	192	13.2	87.4	202	212	17.0	89.9
203	280	17.4	92.5	204	240	13.9	92.7
205	210	11.2	88.8	206	232	14.4	92.3

TABLE II—(continued)

Animal No.	Live weight grams	Protein in milk consumed grams	Coefficient of digestibility	Animal No.	Live weight grams	Protein in milk consumed grams	Coefficient of digestibility
Period 3. April 6 to April 16, 1931							
Brand B evaporated milk				Fresh whole milk			
191	350	15.1	88.0	192	337	17.2	92.3
193	315	16.0	88.1	194	337	18.3	89.6
195	308	13.2	86.6	196	290	15.3	91.6
197	247	20.0	91.2	198	269	22.7	91.9
199	267	16.0	86.9	200	242	18.2	91.3
201	207	16.0	88.2	202	210	18.2	89.8
203	280	11.2	86.9	204	230	12.7	93.6
205	195	13.3	88.8	206	219	15.2	91.5
207	230	13.2	86.4	208	284	15.2	91.0
Period 4. April 28 to May 8, 1931							
Fresh whole milk				Brand B evaporated milk			
191	305	11.9	93.0	192	330	10.2	*
193	328	19.6	93.3	194	332	17.4	86.2
195	309	20.0	90.5	196	307	17.4	86.3
197	269	22.4	91.6	198	290	19.5	87.1
199	297	23.1	90.7	200	260	20.1	85.9
201	198	18.6	93.5	202	224	13.0	86.4
203	272	19.1	92.4	204	222	16.9	88.1
205	175	13.5	92.4	206	225	11.6	86.7
207	216	13.2	89.0	208	277	11.3	81.7
227	222	15.0	90.5	228	229	13.0	84.9

\* Determination lost

\*\* Discontinued—litter born

TABLE III  
COMPARATIVE DIGESTIBILITY OF FAT AND TOTAL SOLIDS OF FRESH WHOLE MILK AND EVAPORATED MILK

Animal No.	Live weight grams	Fat in milk consumed		Total solids in milk consumed	Coefficient of digestibility		Animal No.	Live weight grams	Fat in milk consumed		Total solids in milk consumed	Coefficient of digestibility	
		grams	grams		Fat	Total solids			grams	grams		Fat	Total solids
Fresh whole milk													
155	145	12.4	51.9	99.6	93.8		156	193	15.8	54.2	99.2	92.3	
157 *	113	11.9	49.5	99.7	94.7		160	212	24.6	84.4	98.8	91.6	
189	88	10.0	41.6	99.4	95.4		190	63	12.7	43.5	98.3	93.3	
Brand R evaporated milk													
Fresh whole milk													
163	92	10.8	44.9	99.6	95.0		164	107	13.7	45.9	98.8	92.1	
165	104	12.0	49.9	99.7	95.2		166	136	15.3	51.0	96.4	91.6	
167	114	12.3	51.4	98.8	93.9		168	167	15.7	52.6	98.0	90.5	
171	103	11.2	46.9	99.5	94.3		172	128	14.3	48.0	98.1	90.5	
Brand B evaporated milk													
Fresh whole milk													
173	115	9.1	45.8	99.4	94.5		174	117	14.4	52.0	99.7	95.2	
175	109	8.9	44.6	99.4	94.1		176	125	14.0	50.6	99.2	93.4	
177	118	9.1	45.8	99.4	94.4		178	150	14.4	52.0	99.3	93.7	
179	108	8.9	44.6	99.3	95.2		180	121	14.0	50.6	99.8	95.8	
181	102	8.8	44.1	99.6	95.6		182	133	13.8	50.1	99.4	94.3	
183	100	8.6	43.0	99.3	93.9		184	109	13.5	49.0	99.8	97.1	
185	117	9.1	45.5	99.5	95.4		186	126	14.4	52.2	99.5	94.1	

\* Animal died

14 coefficients for fresh whole milk, only one falls below 99; in an equal number of coefficients for evaporated milk, six fall below 99. Although the coefficients for fat of the pair mates fed Brand "B" evaporated milk are lower than those of the pair mates fed fresh whole milk, the difference between the total group of animals fed Brand "B" and the total group fed whole milk (Table III) is so small that no significance can be attached to it.

Qualitative tests for sugar were made on individual collections of feces of five rats fed fresh whole milk, two rats fed Brand "R" evaporated milk, three rats fed Brand "D" evaporated milk, and upon combined collections of feces from eight animals fed fresh whole milk and eight animals fed Brand "B" evaporated milk. In all cases the tests were negative. The tests for sugar, therefore, lead to the conclusion that the sugar of fresh whole milk and of evaporated milk supplied to rats as the only food is completely digested.

The solids of milk were found to be lower in digestibility than the fat, but not so low as the protein (Table III). This result is in harmony with the data for the individual constituents of the solids, since it would be expected that, with the fat and sugar almost completely digestible, and the protein from 88 to 92 per cent digestible, the coefficients for total solids would fall between those for protein and 100. The coefficients found for the total solids of Brand "B" evaporated milk are significantly lower than those for the solids of fresh whole milk, and the results obtained with two pairs of animals indicate lower coefficients for Brand "R" evaporated milk than for fresh whole milk. In the comparison of Brand "D" evaporated milk and fresh whole milk, however, the results are quite variable. These experiments have not demonstrated as conclusively as might be desired, therefore, an expected lower digestibility of the solids of evaporated milk than of the solids of fresh whole milk.

#### SUMMARY

Two brands of commercial evaporated milk and laboratory evaporated milk were fed in comparison with fresh whole milk in paired feeding trials with albino rats. Digestibility of the milk was studied.

The protein of fresh whole milk was distinctly higher in digestibility than that of either the commercial or the laboratory evaporated milks. Reversal trials with stock animals showed that the lower coefficients for evaporated milk were not related to the individuality of the animals but that each animal digested fresh whole milk more completely than evaporated milk.

The fat of the milk fed was about 99 per cent digestible but no signifi-

cant differences between the digestibility of fat in fresh whole milk and in evaporated milk were demonstrated.

No sugar was found in the feces of rats fed either fresh whole milk or evaporated milk.

Coefficients of digestibility of total solids of milk lay between those for protein and those for fat, but these experiments showed no significant differences between fresh whole milk and evaporated milk in digestibility of total solids.

The differences in digestibility of fresh whole milk and of evaporated milk do not explain fully the differences in the nutritive properties of the two kinds of milk, since the evaporated milk proved superior for growth. This explanation is reserved for a later publication.

#### ACKNOWLEDGMENT

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# A STUDY OF THE ANTIMONY TRICHLORIDE COLOR REACTION FOR VITAMIN A

## V. EVALUATION OF A COLORIMETRIC UNIT ON THE BASIS OF THE BIOLOGICAL UNIT FOR VITAMIN A

By

EARL R. NORRIS AND ANNA E. CHURCH

*(From the Division of Biochemistry, University of Washington, Seattle)*

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RECENT work has shown that the fat-soluble, growth-promoting factors measured as vitamin A, of plant and animal source are not identical chemical compounds. Consequently, any chemical test cannot be applicable to the growth-promoting substance from both sources. However the antimony trichloride test suggested by Carr and Price (1) may be used under certain conditions for the determination of vitamin A in animal oils such as cod liver oil.

In previous papers of this series (2) it has been pointed out that both the colorimetric and biological assays of vitamin A must be carefully standardized in order to obtain consistent and comparable results. As the color produced by the action of antimony trichloride reagent on a cod liver oil is not a linear function of the amount of oil used in the test, comparison of the colors produced by two different oils at any given concentration or between the color produced directly by an oil, and the feeding experiments on the same oil, cannot be compared. Comparison can only be made where the color may be considered as a linear function of the amount of the oil taken for the test. It has been previously shown that the tangent to the dilution curve at the origin may be considered as representing the intensity of color that would be produced if there were no interfering substances present and the color produced were a linear function. In this paper comparison is made with the values calculated from the tangents to the dilution curves.

In making the comparison of the two methods of assay for vitamin A, the biological assay used was based upon that described in U.S.P.X., incorporating such precautions as have been brought out by recent work and are mentioned in the following discussion of the procedure.

Young rats were weaned at 28 to 29 days of age from females of the breeding colony on a diet consisting of:

1000 gm.	ground whole wheat
500 gm.	dried whole milk
75 gm.	wheat germ
75 gm.	dried yeast
20 gm.	sodium chloride (iodized)

The diet was supplemented three times a week with fresh green lettuce leaves, and with about three grams of fresh lean beef per rat.

At the time of weaning the young rats were placed on a vitamin A-free diet, which consisted of:

Purified casein	18 per cent
dried yeast	10 " "
salt mixture	
(Osborne and Mendel)	4 " "
sodium chloride	1 " "
corn starch	67 " "

The casein was purified according to Sherman and Munsell (3), by extracting three times with boiling 95 per cent alcohol. The Osborne and Mendel (4) salt mixture was modified by the addition of 0.161 grams of hydrated copper sulfate for each 134.8 grams of calcium carbonate used, in order to supply the required copper. The dried yeast fed at 10 per cent was shown to be ample as a source of vitamin B when fed as 3 per cent of the diet. Vitamin D was supplied by irradiating the rats at a distance of 27 inches from a quartz mercury vapor lamp three times a week for a period of five minutes during the depletion period, and in a similar manner for a period of ten minutes throughout the experimental period.

The estimation of the conclusion of the depletion period or the time of the first administration of the supplementary source of vitamin A is one of the most important points in obtaining consistent results by the biological method of assay. The vitamin A must be completely depleted from the animal, but if pathological changes due to vitamin A deficiency are allowed to become too severe, the recovery is retarded or entirely prevented upon adding the supplementary oil to be tested. An arbitrary rule, such as seven days of stationary or declining weight, in our experience does not give consistent results; as with some groups the drop from a maximum weight is very rapid with rapid development of severe deficiency symptoms while with other groups the growth curves flatten and weight drops more gradually. We believe that the proper time first to administer the supplementary source of vitamin A cannot be adequately determined by a fixed rule but must be interpreted in the light of the worker's experience in observing the onset of deficiency symptoms in a large number of rats, and checking by running controls to be certain of depleting the stored vitamin. The average

depletion period of 105 rats raised on the above stock diet was 31 days, varying from 26 to 33 days. The rats invariably showed early stages of ophthalmia, and at the time of the first administration of vitamin A had shown stationary weight, on an average as compared to the sixth previous day. The period of stationary and declining weight varied from 5 to 8 days depending upon the condition of the eyes and the gross symptoms of vitamin A deficiency.

The oil to be tested was fed separately from the diet three times a week, as a solution in olive oil, which was free from vitamin A. The solution of cod liver oil in olive oil was prepared fresh every two weeks, to prevent appreciable destruction of the vitamin dissolved in the oil. Several of the olive oil solutions of cod liver oil were tested colorimetrically with antimony trichloride at the beginning and end of the 2 weeks' period during which it was used, and showed no appreciable change in chromogenic substance.

Ten rats were used for each level of oil upon which the comparison was made, litter mates being separated to different levels of oil to equalize possible litter variations. Controls showed the animals to be depleted of vitamin A, and the yeast and olive oil used to be free of vitamin A.

The experimental period was continued for eight weeks and the gain in weight calculated for periods of four, five, and eight weeks. The length of the experimental period has been discussed many times from the standpoint of a balance between the accuracy of the determination and the economic necessity of making the procedure as short as practicable. Representative values obtained with oil U for two levels of feeding are given in Figure 1, which shows a scattering of growth response during the first weeks of the experimental period and the bunching of the results after the fourth and fifth weeks, which was found characteristic for each group. The tendency of growth curves of all rats plotted in Figure 1 was toward an increase in weight or maintenance at the end of the eight weeks' experimental period, with the exception of two animals which contracted a pulmonary infection during the sixth week and lost weight rapidly thereafter. However, no animals were eliminated from the averages used for comparison of methods. The average deviations of growth response from the arithmetical mean for each group of rats on both oils were similar and gave the following average values for successive weeks from the first to the eighth: 3.2, 2.5, 1.6, 1.3, 1.4, 1.1, 1.1, 1.0. The average deviation from the mean became smaller with a longer experimental period. The coefficient of variation decreased with the length of experimental period in any one group of rats, and varied with the amount of cod liver oil fed, as would be



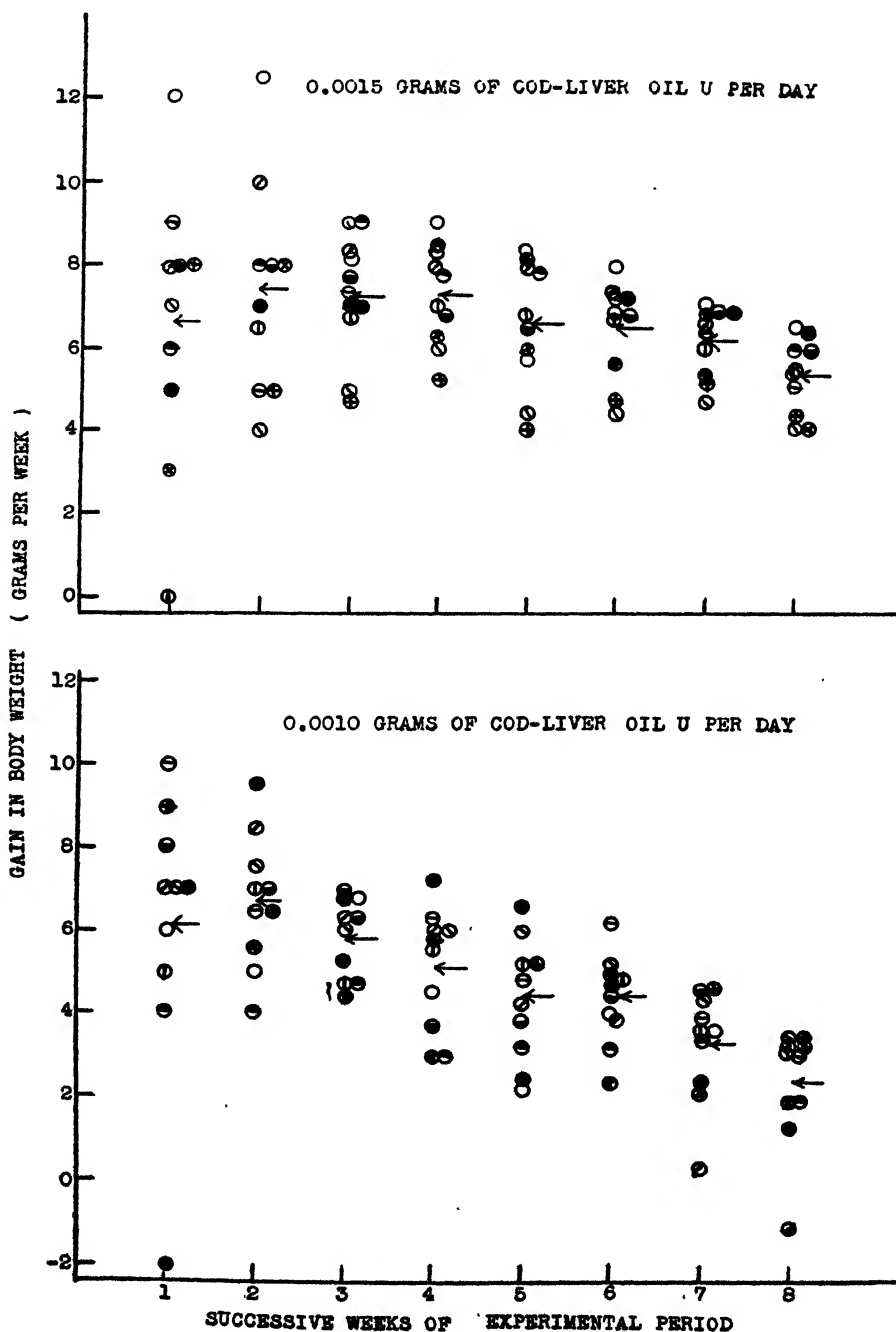


FIG. 1. Weekly gain in weight of rats fed two levels of cod-liver oil U; showing a decrease in variation from the mean, indicated by arrow, with a longer experimental period.

expected, increasing somewhat with a decrease in total gain per week. The incidence of ophthalmia was less consistent than the growth response. With those animals which rapidly developed a marked ophthalmia at the end of the depletion period, and were placed on a low level of cod liver oil, the

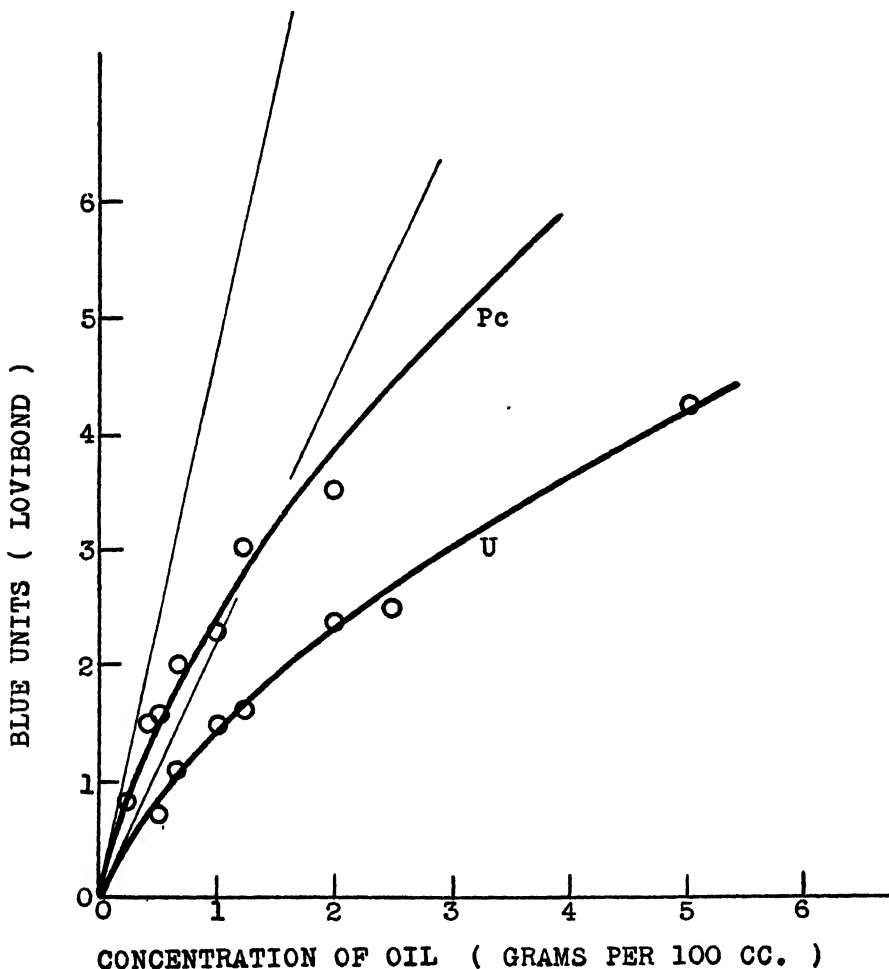


FIG. 2. Dilution curves for cod-liver oils U and Pc.

ophthalmia often persisted for several weeks and in some cases throughout the experimental period, with a tendency to a lower total gain in weight and greater susceptibility to cold and infection.

Table I gives the data for feeding experiments on cod liver oils Pc and U. The values obtained in these tests, where care was taken to insure ample

TABLE I  
FEEDING EXPERIMENTS ON TWO COD LIVER OILS, GIVING THE  
AVERAGE GAIN IN BODY WEIGHT IN GRAMS PER WEEK

Duration of exp. period weeks.	Cod Liver Oil U Oil fed—Grams per day			Cod Liver Oil Pc Oil fed—grams per day		
	0.00075	0.0010	0.0015	0.00037	0.00073	0.0011
4	2.6	5.0	7.3	3.6	6.6	9.1
5	1.6	4.4	6.6	2.3	5.5	8.5
8	2.2	2.4	5.4	0.9	3.9	6.5

vitamin B complex, and to insure against destruction of the fat-soluble factor during the time of feeding, were approximately three times as high as tests previously made in this laboratory with the same colony of rats on the same stock diet, and using the same general technic. Peanut oil, which has been shown to be more destructive to vitamin A, was used as a diluting oil in the previous tests.

Figure 2 gives the continuous curves as calculated from the experimental data using the formula  $X = aY^2 + bY$  for the two cod liver oils U and Pc. The circles represent the observed values for the respective oils. The tangents to the curves calculated as  $dX/dY$  where Y equals 0 are also shown.

A correlation of the feeding experiments with the colorimetric values calculated from the tangents to the dilution curves on the two cod liver oils tested is given in Table II. The values obtained show a fraction of a

TABLE II  
LOVIBOND BLUE UNITS, ESTIMATED FROM THE TANGENTS TO THE  
DILUTION CURVES, EQUIVALENT TO TEN BIOLOGICAL UNITS

Duration of exp. period weeks	Cod Liver Oil U	Cod Liver Oil Pc	Average
4	4.5	4.0	4.2
5	5.5	5.7	5.6
8	7.1	8.2	7.6

blue unit for one biological unit, and in order to make the comparison on the basis of higher values of blue units, the results were calculated to a basis of 10 biological units. A biological unit is considered to be that amount of oil required per day to induce an average body weight increase of 3 grams per week for a definite period of time; as four, five, or eight weeks, when fed under the conditions outlined above.

## SUMMARY

A correlation can be made between the colorimetric assay for vitamin A using antimony trichloride, if the color value is compared where it is a linear function of the cod liver oil used in the test, and the biological assay.

Colorimetric units have been calculated in terms of the biological units for vitamin A.

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# THE NUTRITIVE VALUE OF CERTAIN ANIMAL PROTEIN CONCENTRATES\*

By

P. B. CURTIS, S. M. HAUGE AND H. R. KRAYBILL

*(From the State Chemist Department and Research Chemical Laboratory, Purdue University Agricultural Experiment Station, Lafayette, Indiana)*

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THE value of supplementing home grown grains with protein concentrates is well recognized. Animal tankages and meat scraps are used extensively as protein supplements in rations for swine and poultry. These products furnish essential amino acids in which the home grown grains are deficient. It is customary to evaluate these protein concentrates on the basis of their content of crude protein although there is much evidence available that this is not a true index of their value.

Feeding tankages and meat scraps are prepared from inedible portions of slaughtered animals and the entire carcasses of diseased and dead animals. After rendering them, to extract as much of the fat as possible, the residues are used as feeds. Kraybill (1) described the types of raw material and the processes used in their manufacture. Hoagland and Snider (2, 3, 4) have shown that there is a wide difference in the nutritive value of the proteins of various organs and tissues of the animal. Mitchell and Carman (5) and Mitchell, Beadles and Kruger (11) obtained similar results and showed that the proteins of meats containing larger amounts of connective tissue are inferior in nutritive value. This is probably due to the fact that the proteins of connective tissues are high in collagen which yields gelatin, which is recognized as being deficient in tyrosine, cystine and tryptophane. It is evident from the wide variety of materials used in their manufacture, that tankages of the same crude protein content do not necessarily have equal feeding value.

## EXPERIMENTAL

It seemed possible that a determination of the amount of hot water soluble protein ( $N \times 6.25$ ) in tankages and meat scraps might be helpful

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\* Part of these data are from a thesis presented to the Graduate School of Purdue University by Mr. P. B. Curtis in partial fulfillment of the requirements for the degree of Master of Science. Presented before the Division of Agriculture and Food Chemistry at the Indianapolis Meeting of the American Chemical Society, March 31, 1931.

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in determining their nutritive value. Consequently, we have determined the amount of water soluble protein in a number of commercial tankages and meat scraps. In addition a study was made of the nutritive value of two of the original products and their water soluble and insoluble fractions when used as a supplement to corn.

### *Variation in Water Soluble Protein Content*

The following method was used to determine the water soluble protein:

Two and one half grams of the product are dried and extracted with ether to remove the fat. The sample is then transferred to a filter paper and washed with successive portions of boiling water until 250 cc. are obtained. Protein ( $N \times 6.25$ ) is then determined on an aliquot of the filtrate and also upon the insoluble residue by the official Gunning method (6).

Analyses of a number of samples of different brands of tankages and meat scraps secured on the market show considerable variation ranging from 19.6 to 45.8 per cent of the total protein as water soluble (Table I). This is not surprising since the protein contents of tankages are built up by the addition of blood or "stick"<sup>1</sup> or a combination of both of them. Dried whole blood or dried coagulated blood is low in water soluble protein while "stick" is almost entirely soluble in boiling water. Consequently the amount of water soluble protein in a tankage is dependent upon the relative amounts of "stick" and blood used in building up the protein content. Reduction tankages on the other hand are not built up with "stick." Consequently, they are lower in total protein and in percentage of water soluble protein.

A number of samples of two brands of digester tankages manufactured by two different packing houses over a period of two years were examined. One brand shows a variation of from 32.7 to 45.8 per cent of the total protein as hot water soluble and the other a range of from 23.2 to 44.0 per cent. Examination of these samples shows that those having the lowest percentages of hot water soluble protein contain the most blood while those having the higher percentages contain more "stick."

Meat scraps are dry rendered and are not built up in protein content by the addition of "stick" or blood. Since they are dry rendered the water soluble proteins are not removed from the tissues in processing. Analyses of these products range from 26.9 to 40.3 per cent of the total protein as water soluble (Table I). Super meat scraps or the products containing around 75 per cent total protein contain the highest amounts of water

<sup>1</sup> "Stick" is the product resulting from the concentration of the liquors obtained in the wet rendering process of tankage products.

TABLE I  
DISTRIBUTION OF NITROGEN OF VARIOUS MEAT BY-PRODUCTS

Product	Sample No.	Nitrogen	Crude protein N×6.25	Distribution of Total N		
				Hot water insoluble N	Hot water soluble N	Ether extract N
		Per cent	Per cent	Per cent	Per cent	Per cent
Digester tankages or	1	9.760	61.0	60.7	36.5	0.65
meat meal tankages	2	9.936	62.1	63.9	34.5	0.42
" "	3	10.048	62.8	53.0	44.9	0.44
" "	4	9.792	61.2	70.9	27.3	0.42
" "	5	10.136	63.3	64.9	32.7	0.47
" "	6	9.958	62.2	54.0	45.8	
" "	7	10.043	62.6	61.5	38.2	
" "	8	9.862	61.6	65.8	34.1	
" "	9	9.813	61.3	63.9	35.9	
" "	10	10.345	64.7	63.5	36.0	
" "	11	9.975	62.3	57.0	42.7	
" "	12	9.868	61.7	68.1	31.5	
" "	13	9.678	60.5	71.2	28.4	
" "	14	10.009	62.6	76.3	23.2	
" "	15	10.003	62.5	55.3	44.0	
Meat scraps	16	8.128	50.8	71.8	26.9	0.41
" "	17	8.160	51.0	67.5	29.9	0.44
Super meat scraps	18	12.112	75.7	59.5	39.2	0.32
" "	19	12.416	77.6	58.7	40.3	0.32
Dried whole blood	20	11.958	74.7	96.7	2.3	0.09
Dried coagulated blood	21	11.912	74.5	89.8	8.5	0.14
" "	22	11.873	74.2	91.9	6.4	0.23
Reduction tankage	23	7.467	46.7	77.6	19.6	
" "	24	8.735	54.6	76.4	20.9	



soluble protein. These high protein meat scraps are composed largely of the degreased cracklings resulting from the rendering of adipose tissues. These tissues are high in collagen and hence yield large amounts of water soluble proteins.

### *Nutritive Value of Meat Meal Tankage*

Twenty pounds of a commercial meat meal tankage which was composed of dry rendered meat and bone scraps and blood were separated into a water insoluble and a water soluble fraction. Both fractions were then dried in a current of warm air. A sample of commercial "stick" was secured and dried in a similar manner. Each of these products was fed to albino rats as a sole source of protein and as a protein supplement to corn. The analyses of these products are shown in Table II.

TABLE II  
CHEMICAL COMPOSITION OF EXPERIMENTAL SAMPLES USED IN RATIONS

Product	Moisture	Ash	P <sub>2</sub> O <sub>5</sub>	Crude protein N×6.25	Hot water soluble protein; % of total protein
	Per cent	Per cent	Per cent	Per cent	Per cent
Meat meal tankage (fat free)	3.90	21.50	7.98	72.80	28.0
Hot water insoluble fraction	2.50	29.50	11.95	64.40	5.4
Hot water soluble fraction	6.00	9.30	0.82	90.49	98.3
Commercial "stick" dried	2.40	11.30	1.80	80.34	97.2
Bone ash			39.66	0.24	
Dried yeast				47.54	
Yellow corn				9.39	
Wheat bran				17.60	
Digester tankage (fat free)			8.16	69.30	32.7
Hot water insoluble fraction			11.72	62.30	
Hot water soluble fraction			1.02	82.70	
Tricalcium phosphate			43.60		
Vitamin B concentrate				43.00	

These products were supplemented with other factors to make a satisfactory ration with the possible exception of the protein moiety. Since the ration containing the insoluble fraction contained the highest percentage of ash, the other rations of each group of tests were equalized by the addition of bone ash to give the same phosphoric acid content. Two per cent of McCollum's No. 185 salt mixture was also included in the ration. Eight per cent butter fat, two per cent cod liver oil and three per cent dried yeast

TABLE III  
COMPOSITIONS OF RATIONS IN MEAT MEAL TANKAGE SERIES

Lot. No.	Protein Combinations			Ingredients of Ration												
	Animal	Corn	Wheat bran	Butter fat	C.L.O.	Dried yeast	Salt mixt. 185*	Agar	Bone ash	Meat meal tankage	Insol. fract.	Sol. fract.	Stick	Yellow corn	Wheat bran	Dex- trin
Type	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
1	Meat meal	15		8.0	2.0	3.0	2.0	2.0	2.88	20.6	23.30					59.52
2	Insoluble	15		8.0	2.0	3.0	2.0	2.0	2.0							59.70
3	Soluble	15		8.0	2.0	3.0	2.0	2.0	6.67			16.50	18.68			59.83
4	Stick	15		8.0	2.0	3.0	2.0	2.0	6.17							58.15
5	Meat meal	9	6	8.0	2.0	3.0	2.0	2.0	4.53	12.36				63.83		2.28
6	Insoluble	9	6	8.0	2.0	3.0	2.0	2.0	2.81		13.98			63.83		2.38
7	Soluble	9	6	8.0	2.0	3.0	2.0	2.0	6.81			9.94		63.83		2.42
8	Stick	9	6	8.0	2.0	3.0	2.0	2.0	6.51				11.21			1.45
9	Meat meal	10		8.0	2.0	3.0	2.0	2.0	2.54	13.74						66.72
10	Insoluble	10		8.0	2.0	3.0	2.0	2.0	0.62		15.53					66.85
11	Meat meal	4	6	8.0	2.0	3.0	2.0	2.0	4.19	5.50						9.48
12	Insoluble	4	6	8.0	2.0	3.0	2.0	2.0	3.43		6.21					9.53
13	Soluble	6	6	8.0	2.0	3.0	3.0	2.0				6.60		63.8		11.60
14	Soluble	4	6	8.0	2.0	3.0	3.0	2.0				4.40		63.8	11.4	2.40
15			6	8.0	2.0	3.0	3.0	2.0						63.8	11.4	6.80

\* McCollum, E. V. and Simmonds, N., *Jour. Biol. Chem.*, 1918, 33, 63.

supplied sufficient quantities of vitamins A, B and D. The remainder of the ration consisted of two per cent agar-agar and sufficient dextrin to complete the ration. The composition of the rations is given in Table III.

Each fraction was fed both as a sole source of protein at a level of 15 per cent and also as a supplement to corn protein (9 per cent from tankage fraction and 6 per cent from corn). The meat meal tankage and insoluble

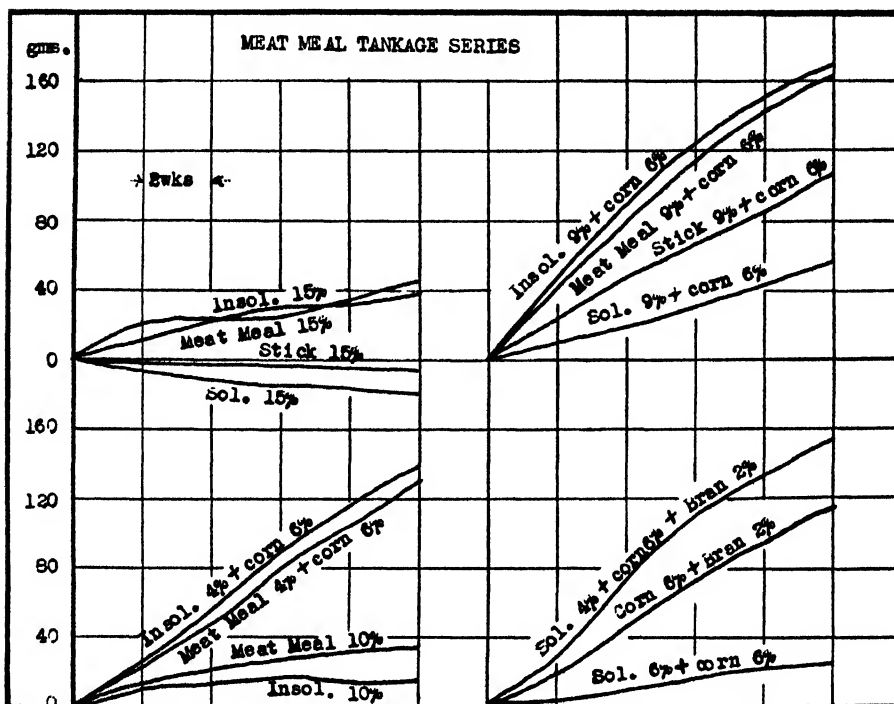


CHART 1. Composite growth curves of groups of rats showing the relative nutritive value of meat meal tankage and its fractions at various protein levels.

fraction were also fed, as sole sources of protein at levels of 10 per cent and as supplements to corn protein (4 per cent from meat meal tankage or insoluble fraction and 6 per cent from corn). Three male and two female albino rats were used in testing each ration. The duration of each test was ten weeks.

The results of these feeding tests are shown in Chart 1. Each growth curve represents a composite of the growth of the rats of each lot. The lots receiving meat meal tankage and the hot water insoluble fraction as sole sources of protein at levels of 10 and 15 per cent of protein made very poor growth. The average rate of gain at the 15 per cent level was almost identi-

cal with both products. At the 10 per cent level the meat meal was slightly superior to the insoluble fraction.

When these products were used to supplement corn in rations containing 6 per cent protein from yellow corn and either 9 or 4 per cent protein from the meat meal tankage, or the insoluble fraction, the animals made fairly good gains. In both instances the insoluble fraction was slightly superior to the original product.

The hot water soluble fraction or "stick" fed as a sole source of protein at a 15 per cent level of protein gave very poor results. The ration was not even adequate for maintenance. Four of the five animals in the lot fed with the ration containing the water soluble fraction died before the end of the ten weeks' test. As a supplement to the protein of corn (water soluble fraction protein or "stick" protein 9 per cent and corn protein 6 per cent) the water soluble fraction and "stick" were only moderately satisfactory. Under these conditions "stick" gave definitely better results than the water soluble fraction. This difference may be due to the fact that commercial "stick" contains some suspended insoluble material.

The results show very definitely that the original meat meal tankage and the insoluble fraction are much more effective than "stick" or the water soluble fraction in supplementing the proteins of corn. Other experiments not reported here show that both dried whole blood and coagulated blood are much superior to "stick" and the soluble fraction as a supplement to the proteins of corn.

Since the hot water soluble fraction is not effective in supplementing the protein of corn, studies were made to determine the amino acid deficiencies of this combination. The ration containing 6 per cent corn protein and 6 per cent soluble fraction protein proved to be unsatisfactory for growth although adequate for maintenance. Since the water soluble fraction consists very largely of gelatin which is recognized as being deficient in tyrosine, cystine and tryptophane, various combinations of these amino acids were added to this ration. The amounts introduced in the basal ration were 0.30 per cent cystine, 0.30 per cent tyrosine and 0.18 per cent tryptophane. The results of these tests are given in Table IV.

These results show that the addition of the three amino acids, tryptophane, cystine and tyrosine, to the basal ration containing 6 per cent corn protein and 6 per cent soluble fraction protein resulted in marked increase in growth. The omission of tyrosine only did not apparently affect the nutritive value of the ration. However, by omitting cystine only there was a decreased growth rate, but if tryptophane was omitted, the ration even though supplemented with tyrosine and cystine was but little better than

the basal ration alone. These results show that the main deficiency of the basal ration is tryptophane, cystine to a lesser degree and that a deficiency in tyrosine does not exist.

When two per cent protein from the soluble fraction was substituted by sufficient wheat bran, which contains tryptophane, to furnish two per cent protein there was a very marked improvement in the basal ration (Chart 1). This ration containing 12 per cent protein (soluble protein 4 per cent, corn protein 6 per cent and bran protein 2 per cent) resulted in satisfactory growth while the basal ration (6 per cent soluble protein and 6 per cent corn protein) was little more than adequate for maintenance. The substi-

TABLE IV  
RESULTS OF SUPPLEMENTING THE SOLUBLE FRACTION OF MEAT MEAL AND CORN WITH CERTAIN AMINO ACIDS

Lot No.	Period 1 3 weeks		Period 2 4 weeks		Period 3 3 weeks	
	Ration	Weekly gain	Ration	Weekly gain	Ration	Weekly gain
16	Basal*	2	Basal*	3	Basal*	3
17	Basal	2	Basal+Trypt.+Cyst.+Tyr.	14	Basal+Cyst.+Tyr.	7
18	Basal	3	Basal+Trypt.+Cyst.	14	Basal+Cyst.	7
19	Basal	3	Basal+Trypt.+Tyr.	9	Basal+Tyr.	5
20	Basal	3	Basal+Cyst.+Tyr.	4	Basal+Cyst.+Tyr.	2

\* See Table III, Lot No. 13.

tution of wheat bran equivalent to two per cent protein for the entire six per cent of water soluble protein, in the basal ration resulted in improvement but not so marked as when only two per cent of the water soluble protein was replaced by bran. These results show that although the water soluble fraction of meat meal tankage has very little supplementing value to corn alone, it does supplement the combined proteins of corn and bran.

#### *Nutritive Value of Digester Tankage*

A sample of commercial digester tankage, prepared by building up a wet rendered product with stick and blood, was separated into a hot water soluble and a hot water insoluble fraction in the same manner as described for meat meal tankage.

The original tankage and each fraction were fed with yellow corn at a protein level of 12 per cent (5 per cent from tankage or its fractions and 7 per cent from corn). Three per cent of butter fat was used and one per cent

TABLE V  
COMPOSITION OF RATIONS IN DIGESTER TANKAGE SERIES

Lot No.	Protein Combinations			Ingredients of Ration										
	Animal	Corn	Wheat bran	Butter fat	C.L.O.	Vita- min B conc.	Salt mixt. 185*	Trical- cium phosph.	Digester tankage	Insol. fraction	Sol. fraction	Yellow corn	Wheat bran	Dextrin
	Type	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent
21	Tankage	5	7	3.0	2.0	1.0	2.0	0.80	7.22			72.16		11.82
22	Insoluble	5	7	3.0	2.0	1.0	2.0			8.02		72.16		11.82
23	Soluble	5	7	3.0	2.0	1.0	2.0	2.01			6.04	72.16		11.79
24			7	3.0	2.0	1.0	2.0	2.15				72.16		17.69
25	Tankage	3	7	3.0	2.0	1.0	2.0	1.34	4.33			72.16	11.3	2.87
26	Insoluble	3	7	3.0	2.0	1.0	2.0	0.86		4.80		72.16	11.3	2.88
27	Soluble	3	7	3.0	2.0	1.0	2.0	2.07			3.63	72.16	11.3	2.84
28			7	3.0	2.0	1.0	2.0	2.15				72.16	11.3	6.39

\* McCollum, E. V., and Simmonds, N., *Jour. Biol. Chem.*, 1918, 33, 63.

of specially prepared vitamin B concentrate<sup>3</sup> was substituted for the 3 per cent yeast used in the rations of the series previously described. Tricalcium phosphate instead of bone ash was used to adjust the mineral content of the rations. The composition of these rations is given in Table V.

The results of these trials are given in Chart 2. The ration containing corn alone (protein 7 per cent) resulted in very poor growth. The addition of water soluble fraction sufficient to furnish five per cent of protein in the ration and to raise the total protein content to 12 per cent resulted in no improvement over the ration containing corn alone. When a sufficient amount of the insoluble tankage fraction or of the original tankage was

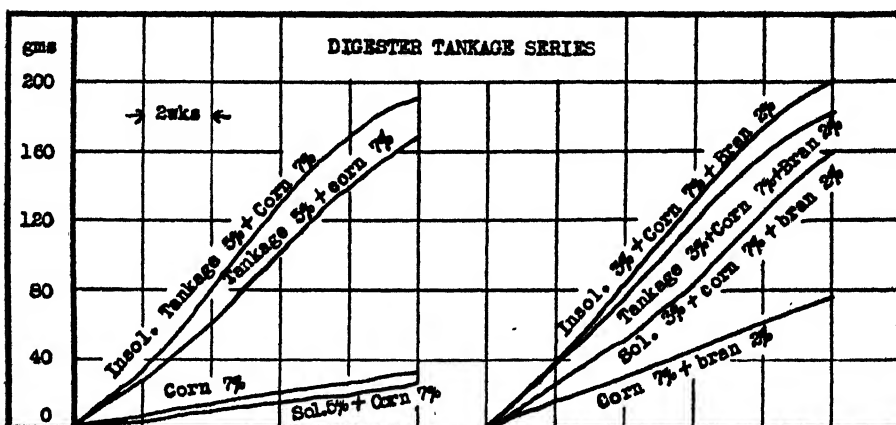


CHART 2. Composite growth curves of groups of rats showing the nutritive value of digester tankage and its fractions in supplementing corn protein and a combination of corn and wheat bran proteins.

added to the ration to furnish five per cent of protein, growth was very satisfactory. These results show that the water soluble fraction of digester tankage does not supplement the protein of corn while the original tankage or the insoluble fraction does supplement the protein of corn. The insoluble fraction is apparently slightly superior to the original tankage as a supplement to corn.

When a sufficient amount of bran to furnish two per cent of protein was added to the ration containing only corn protein, improvement was noted. The further addition of three per cent of protein in the form of the soluble fraction resulted in even more marked improvement. It is thus apparent that while the soluble fraction does not supplement corn alone, it does supplement the combination of corn and bran (corn protein 7 per cent and

<sup>3</sup> Prepared by the method of Osborne and Wakeman, *Jour. Biol. Chem.* 1919, 40, 383.

bran protein 2 per cent). However, both the insoluble fraction and the original tankage are more effective than the soluble fraction in supplementing corn and bran at this level. The insoluble fraction is slightly superior to the original tankage in supplementing corn and bran, but the difference is not so marked as in supplementing corn alone.

TABLE VI  
RESULTS OF SUPPLEMENTING THE SOLUBLE FRACTION OF DIGESTER TANKAGE AND  
CORN WITH CERTAIN AMINO ACIDS AND WHEAT BRAN

Lot No.	Period 1 3 weeks		Period 2 3 weeks		Period 3 3 weeks	
	Ration	Weekly gain	Ration	Weekly gain	Ration	Weekly gain
29	Basal*	0.0	Basal+Trypt.+Cyst.+Tyr.	+5.3	Basal+Cyst.+Tyr.	-1.0
30	Basal	-1.0	Basal+Trypt.+Tyr.	+2.5	Basal+Trypt.+Cyst.	+5.0
31	Basal	-0.5	Basal+Trypt.+Cyst.	+6.0	Basal+Cyst.	-0.5
32	Basal	-1.0	Basal+Cyst.+Tyr.	0.0	Basal+Trypt.	+4.0
	Period 1 8 weeks			Period 2 3 weeks		
	Ration		Weekly gain	Ration		Weekly gain
33	Corn+Wheat Bran 5%      2%		+4.0	Corn+Wheat Bran+Lysine 5%      2%      0.22%		+8.0
34	Corn+Wheat Bran+Sol. Fr. 5%      2%      3%		+6.8	Corn+Wheat Bran+Sol. Fr. 5%      2%      3%		+6.2

\* Basal ration: soluble fraction 4.84 per cent (equal to 4 per cent protein), corn 61.86 per cent (equal to 6 per cent protein), butter fat 8 per cent, C. L. O. 2 per cent, Vit. B. Conc. 1 per cent, McCollum's salt mixture 4 per cent, agar-agar 2 per cent and dextrin 16.3 per cent.

The addition of 0.22 per cent of lysine (calculated to be equivalent to the lysine content of 3 per cent gelatin) to the ration containing corn and wheat bran (corn protein 5 per cent and bran protein 2 per cent) resulted in improved growth (See Table VI, lots 33 and 34). This indicates a deficiency of lysine in this ration. Since the addition of 3 per cent of the soluble fraction improved growth, it is apparent that the effect of the water soluble fraction in supplementing corn and wheat bran is due to its lysine content.

Experiments to determine the amino acid deficiencies of the corn and water soluble digester tankage fraction were carried out similar to those



described for the meat meal tankage. Practically the same basal ration was used except that the soluble fraction was reduced from 6 per cent to 4 per cent and 1 per cent of the vitamin B concentrate was used in place of the 3 per cent dried yeast. The results (Table VI) show that although slower gains were made, the relative trend in growth of the lots receiving the different amino acids was the same as with the trials with the soluble fraction from meat meal tankage. The results show that the first deficiency of the ration is tryptophane and that when it is corrected, a deficiency in cystine becomes apparent. No indications of a deficiency of tyrosine are evident.

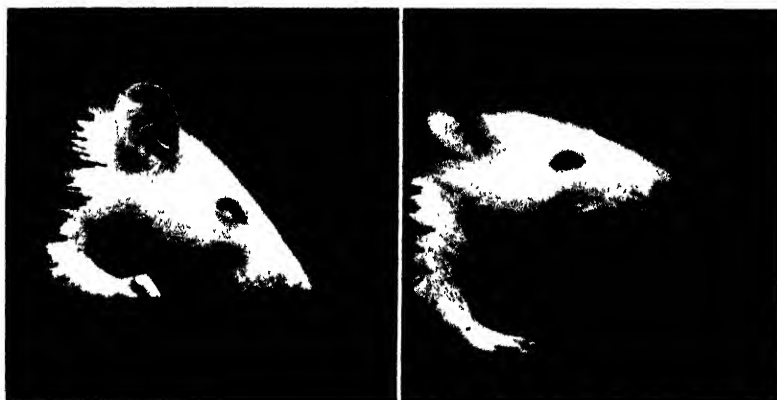


FIG. 1. Showing a type of blindness occurring in rats fed on diets low in tryptophane. This blindness is characterized by opaqueness and loss of the characteristic eye colors and is quite different from ophthalmia due to vitamin A deficiency.

An interesting observation was made that the rats receiving inadequate protein from either low corn protein (Lot 24, Table V) or low protein of the basal ration in the absence of additions of tryptophane developed permanent blindness. This blindness was characterized by white opaqueness of the eye and loss of the characteristic colors of the eye, quite different from ophthalmia due to vitamin A deficiency (Fig. 1). All of the animals in lot 32 (Table VI) became totally blind about the sixth or seventh week. In lot 31 when tryptophane and cystine were added to the basal ration during the second period, and then tryptophane omitted in the last period, only one case of blindness was recorded. This particular rat lost the use of one eye near the end of the eighth week. The other animals in this lot, as well as those in lots 29 and 30, failed to develop this eye condition in the nine weeks' feeding test. Since this blindness occurred only in lots lacking in

tryptophane, this indicates that this condition may be due solely to a tryptophane deficiency.

### *Discussion*

A marked difference was found in the value of the hot water insoluble and soluble fractions of tankages when used as a protein supplement to corn. The soluble fractions had no supplementing value to the proteins of corn while the insoluble fractions supplemented corn and were slightly superior to the original tankages. The inadequacy of the soluble fractions in supplementing corn is due to deficiencies in tryptophane and cystine.

If wheat bran, which furnishes tryptophane and some cystine, is added to the ration containing corn as the sole source of protein the addition of the water soluble tankage fraction gives a marked improvement in the growth of rats. This supplementing action of the water soluble fraction must be due to its lysine content.

When used as a supplement to corn at total protein levels of 15, 12 and 10 per cents, the insoluble tankage fractions are slightly more efficient than the original tankages. That the superiority of the insoluble fractions is due to their higher content of tryptophane and possibly cystine, is indicated by the fact that when the insoluble fractions and the original tankages are used to supplement corn and bran, their differences in effectiveness are less marked.

The results indicate that tankages of low, hot water soluble protein contents may be more effective as supplements to corn per unit of protein than those containing larger amounts of water soluble protein. "Stick" and the water soluble fraction of tankages when used alone to supplement the proteins of corn have little or no value. If some other source of protein, like wheat bran for instance, which contains tryptophane and cystine, is added to the corn the addition of "stick" or the water soluble fraction may have a supplementing action by means of its lysine content.

Feeding experiments with chicks, at this experiment station, have demonstrated that considerable variation exists in the nutritive value of animal protein concentrates. It was found that meat meals (super meat scraps) and digester tankages are inferior to meat scraps when fed at the same protein levels (7, 8, 9). Data were also secured which indicated that the amino acid deficiency of this meat meal (super meat scraps consisting of degreased adipose tissue) was tryptophane (10). Concerning the nutritive value of meat scraps, Prange, Carrick, and Hauge (7) state:

Our experiments suggest that variations exist in the nutritive value of the proteins from meat and bone scraps made by different manufacturers. The cause of this variation is not definitely known, but we surmise it may be due to differences in both amounts and kinds of tissues included in the

product. The variation not only exists in similar products made by different manufacturers, but it is possible that it may exist in different samples of the same brand.

From the work of Mitchell, Beadles, and Kruger (11), it is probable that the relative amounts of connective tissue in the samples may determine its nutritive value. In fact, the addition of connective tissue seems to act as a diluent, reducing the nutritive value of the sample containing proteins of high biological value. This was also emphasized by Hauge (12) who stated that, "Added non-supplementing proteins really act as diluents for the original proteins of the concentrates." In conducting feeding trials on farm animals with animal protein concentrates it therefore appears important to consider the types of materials used in their manufacture.

#### SUMMARY

1. Chemical and biological studies have been made to determine the nutritive value of certain animal protein concentrates.

2. The commercial digester tankages analyzed varied considerably in content of hot water soluble protein ( $N \times 6.25$ ) ranging from 27.3 to 45.8 per cent of the total protein.

3. Commercial meat scraps (dry rendered and containing about 50 per cent total protein) and reduction tankages to which no "stick" or blood are added are usually lower in hot water soluble protein than digester tankages.

4. Super meat scraps (75 to 80 per cent protein) consisting largely of degreased adipose tissue are high in hot water soluble protein (40 per cent of the total protein).

5. Dried whole blood and coagulated blood are low in hot water soluble protein (2.3 to 8.5 per cent of the total protein).

6. As a sole source of protein the hot water soluble fraction of tankages at a 15 per cent protein level is inadequate even for maintenance. The soluble fraction and commercial "stick" have little or no value when used as a supplement to the protein of corn alone, due to a deficiency in the ration of tryptophane and cystine. However, due to their lysine content, these soluble fractions may have some value in supplementing a combination of the proteins of corn and wheat bran.

7. As a sole source of protein at a 15 per cent level, the original tankages and the water insoluble fractions do not support satisfactory growth. As a supplement to corn they are both satisfactory but the insoluble fraction is slightly superior to the original tankage. When used as a supplement to corn and wheat bran, the superiority of the insoluble fraction over the original tankage is less marked.

8. A type of blindness in rats which is different from ophthalmia resulting from vitamin A deficiency, is described. This condition appears to be due solely to tryptophane deficiency.

9. A determination of the amount of hot water soluble protein in tankages is of some help in determining their nutritive value.

10. In conducting experiments to determine the feeding value of animal tankages and meat scraps the type of materials used in their manufacture should be considered.

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## THE EFFECT OF FIGS AND SMALL AMOUNTS OF RAISINS ON URINARY ACIDITY

BY LAWRENCE G. SAYWELL

*(From the Fruit Products Laboratory, University of California,  
College of Agriculture, Berkeley)*

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**I**N A previous paper (3) the effect of fresh Malaga grapes, several kinds of grape juice, grape concentrate, and two varieties of raisins on the urinary acidity was shown. In the previous experiments 1000 cubic centimeters of grape juice or the approximate equivalent of 300 grams of concentrate or raisins was employed. Since the effect of these quantities on the urinary acidity was very marked, it seemed desirable to ascertain the effect of daily ingestion of a smaller amount of raisins. In addition, the study was extended to include the effect of the common white Calimyrna fig, which is a strain of the Smyrna variety.

The experiments were of eleven to twelve days' duration. Each experiment was divided into two consecutive periods. For the first five days each person received only the basal ration. During the next six or seven days each person received, in addition, a constant quantity of raisins or figs. Three normal young men subjects were employed in each experiment.

The basal diet was the same as that described in the previous report (3) with the exception of the bread ration. In the raisin experiment, a standard white bread, similar to that used in the previous studies was employed. However, during the second part of the experiment the raisins were incorporated in the bread. Consequently only 235 grams of bread were eaten daily during the first five days while the body attained equilibrium on the highly acid diet. The raisins were added to the bread in such proportion that each one pound loaf contained five ounces of raisins. Each individual then received 340 grams of raisin bread. That is, each 340 grams of bread contained 105 grams of the seedless raisins, the difference of 235 grams corresponding to the amount of bread eaten daily by each individual during the first five days.

In order that the data would be comparable to those in the previous studies, 300 gram portions of the figs were eaten daily by each individual during the second part of the fig experiment.

All analytical determinations were made in duplicate or triplicate, the average of closely agreeing duplicates only being reported. The methods were the same as those described in the previous study.

TABLE I  
EFFECT OF RAISINS IN BREAD ON COMPOSITION OF URINE

Days*	Subject D					Subject K					Subject L				
	Volume cc.	pH	0.1-N Organic acids cc.	0.1-N Titratable acidity cc.	Ammonia N mg.	Volume cc.	pH	0.1-N Organic acids cc.	0.1-N Titratable acidity cc.	Ammonia N mg.	Volume cc.	pH	0.1-N Organic acids cc.	0.1-N Titratable acidity cc.	Ammonia N mg.
1	930	6.05	538	85	156	1720	6.30	715	21	218	1480	6.45	780	12	148
2	720	5.70	475	130	165	1450	6.25	725	23	203	1850	6.40	820	15	178
3	880	5.70	710	169	173	1230	6.00	718	39	179	660	6.15	640	53	92
4	880	5.70	662	166	173	1210	6.10	735	58	190	790	6.15	695	73	95
5	780	5.70	690	169	179	1150	6.00	713	74	206	1120	5.95	705	103	112
6	770	5.80	620	157	134	1050	5.95	680	118	218	1010	5.75	731	97	109
7	780	5.85	621	103	127	1220	6.40	658	78	171	840	6.25	692	91	97
8	790	6.00	616	98	120	1290*	6.35	676	98	196	900	6.20	735	83	90
9	730	6.00	576	117	114	1210	6.40	696	82	176	1400	6.55	773	62	101
10	940	6.05	586	127	121	1290	6.50	655	67	174	770	6.55	744	62	92
11	780	6.20	575	131	105	820	6.45	630	62	147	1140	6.45	702	64	96
12	770	6.15	575	129	108	1030	6.45	682	53	144	850	6.45	653	37	85
13	770	6.25	572	123	104										

\* Days, 1-5 inclusive, basal diet  
Days, 6-12, 13 inclusive, basal + 105 grams Thompson Seedless Grapes.

TABLE II  
EFFECT OF CALIVERNA FIGS ON COMPOSITION OF URINE

Days*	Subject A					Subject D					Subject M				
	Volume cc.	pH	0.1-N Organic acids cc.	0.1-N Titratable acidity cc.	Ammonia N mg.	Volume cc.	pH	0.1-N Organic acids cc.	0.1-N Titratable acidity cc.	Ammonia N mg.	Volume cc.	pH	0.1-N Organic acids cc.	0.1-N Titratable acidity cc.	Ammonia N mg.
1	1510	6.40	750	70	177	1120	6.20	700	101	238	1060	6.60	649	42	156
2	1500	6.45	726	69	156	1090	6.40	637	122	205	1020	6.40	645	121	215
3	1050	6.10	570	116	170	960	6.00	588	190	216	660	5.95	663	123	200
4	970	6.00	667	103	126	1160	5.95	699	182	208	920	6.05	617	170	170
5	740	5.65	587	130	176	1230	5.90	528	149	210	810	6.00	674	135	193
6	1120	5.85	756	171	180	940	6.40	577	59	171	1020	6.70	654	24	93
7	1060	5.85	646	140	89	1070	6.50	604	74	98	870	6.65	687	54	85
8	2270	6.45	880	91	111	1110	6.65	596	86	70	830	6.70	647	62	78
9	1190	6.55	771	73	68	1100	6.70	706	41	62	1010	6.85	671	27	57
10	1350	6.90	788	36	73	1090	6.85	718	10	61	990	6.85	674	29	56
11	1220	6.85	794	20	64	810	6.80	631	8	46	980	7.00	670	17	42

\* Days, 1-5 inclusive, basal diet  
Days, 6-11 inclusive, basal + 300 grams figs.



In addition to the urine analyses, determinations of the ash content, the alkalinity of the soluble ash, and the alkalinity of the insoluble ash were made (1) upon composite samples of the raisins and figs.

### *Results and Discussion*

As is shown in Tables I and II, the effect of ingestion of the raisins in bread and of figs on the pH of the urine was quite marked. On the basal diet the pH generally stabilized at 5.90 to 5.70. However, subject K (Table I) did not go below a pH of 5.95 while subject A (Table II) dropped down to a pH of 5.65 on the fifth day of the basal ration. This range compares well with the range observed in the previous experiments.

With the raisins there was a generally uniform increase of pH following their addition to the diet. This increase was such that the pH on the last three or four days of the experiment was from 0.45 to 0.6 pH unit higher than that of the fifth or sixth day of the experiment. This compared with the similar change of 0.8 to 0.9 of a pH unit produced in the previous studies by 300 grams of raisins. That is, the smaller amount of raisins also produced a considerable change in the pH of urine. In fact, their efficiency appeared to be increased since only approximately one-third the amount of raisins produced more than half as great a change in pH.

The figs also produced generally uniform increases of the urinary pH. This increase was from 0.9 to 1.1 pH units in magnitude. This change appears to be slightly greater than that of 0.8 to 0.9 of a pH unit produced by the same weight of raisins.

In this respect it is interesting to note the similarity in the ash analyses of the raisins and the figs employed in these experiments. The ash content of the raisins was 2.20 per cent, while that of the figs was 2.52 per cent of the edible material. A slight difference in the relations of the total alkalinities of the ash, however, was observed. That is, the total alkalinity of the ash of the raisins was slightly greater, being equivalent to 339.0 cubic centimeters of tenth normal hydrochloric acid per 100 grams raisins, while that of the figs was equivalent to 328.5 cubic centimeters of tenth normal hydrochloric acid. It is apparent that there is a relation between the change of pH of the urine and the alkalinity of the food material eaten. The greater change of pH appears to be the result of the ingestion of larger quantities of the given food, or of similar quantities of a food of approximately equivalent alkaline ash content. This relation may not be one of direct proportion when differences of as much as 300 per cent occur in the total alkalinity of the ash of the food ingested. It is further possible that there is

a certain optimum range of total alkaline content beyond which the body does not utilize the alkaline factors with complete efficiency.

From the data in Tables I and II showing the values for total acids and ammonia excreted daily, it is evident that there was a decrease in their concentration when the raisins were added to the basal diet. A much more marked decrease occurred with the addition of the figs to the basal diet.

The evaluation of the quantitative relationship between the excretion of acid in excess of fixed bases, as measured by determining the ammonia and titratable acid, and the carbon dioxide binding power of the blood plasma has been developed by Fitz and Van Slyke (2). In the previous study with grapes and grape products this method of estimating the lowering of the alkaline reserve was employed. It was shown that the basal diet alone lowered the alkaline reserve and the grapes and grape products assisted in securing a high alkaline reserve. From a comparison of the data in Tables I and II with those of the previous study, it is evident that the smaller quantity (105 grams) of raisins restored the alkaline reserve of an individual to approximately what it had been at the beginning of the basal diet. In the case of the figs, the 300 grams had increased the alkaline reserve of the subject beyond that obtaining at the beginning of the basal diet period. This was indicated approximately by the smaller values of the ammonia and the titratable acidity titrations. The raising of the alkaline reserve by the 300 grams of figs was of the same order of magnitude as was that produced by a similar weight of raisins (3).

The changes of the pH are of further interest when the percentage oxidation of the organic acids is considered. This follows from the observation that with only 105 grams of raisins complete oxidation of organic acids was noted, while the pH change was from 50 to 60 per cent of that obtained with 300 grams of raisins. With 300 grams of raisins an average of only 92.8 per cent oxidation of the organic acids ingested was noted.

The organic acid content of the raisins and figs was determined by the method of Van Slyke and Palmer (4). The total amounts daily ingested (as cubic centimeters tenth normal acid) are given in Table III. The differences of the average daily organic acid titration of the urine with and without the raisins or figs added to the basal diet are also given. From these values the percentage of the organic acids oxidized in the body may be computed.

It is shown in Table III that the average daily organic acid titration for each individual was less after the raisins were added to the diet than when on the basal ration alone. Considering the organic acids of the added raisins only, one hundred per cent oxidation was observed. This contrasts

with the average of 92.8 per cent oxidation observed in the previous studies with 300 grams of raisins. No explanation is given at this time for the actual average decrease of organic acids occurring on the addition of the raisins.

It is further evident that a slightly higher percentage of oxidation of organic acids from the figs was obtained than was the case with an equal weight (300 grams) of seedless raisins. On the average, 97.5 per cent of the organic acids of the figs was oxidized while the corresponding value for

TABLE III  
ORGANIC ACIDS INGESTED AND OXIDIZED  
(As cc. N/10 HCl)

	Raisins in bread			Calimyrna Figs		
	D	K	L	A	D	M
Average daily organic acid titration for basal diet period	639	721	728	680	630	649
Average daily organic acid titration for basal diet + raisins or figs	593	668	719	772	639	667
Difference	*-46	*-53	*- 9	+92	+ 9	+18
Organic acids ingested daily from raisins of figs.	714	714	714	1620	1620	1620
Per cent oxidation of organic acids	100	100	100	94.3	99.4	98.9

\* Negative values indicate lower organic acid content with raisins added to the diet.

raisins was 92.8 per cent. It is possible that this difference was a result of the lower organic acid content of the figs. The total organic acid content of the 300 grams of figs was equivalent to 1620 cubic centimeters of tenth normal hydrochloric acid while that of the 300 grams of seedless raisins used in the previous studies was equivalent to 2160 cubic centimeters of tenth normal hydrochloric acid. The organic acid content of the figs was therefore only approximately 75 per cent of that of the seedless raisins. Also the fact that there was complete oxidation of the organic acids resulting from only 105 grams of raisins must be considered. Consequently it would appear that for considerable quantities of natural grape and fig products the body is capable of completely oxidizing the organic acids. For larger quantities of the raisins only 92 to 95 per cent of the organic acids ingested may be oxidized.

## SUMMARY

Experiments with men subjects on a basal diet and on the same basal supplemented by Thompson seedless raisins and Calimyrna white figs are reported. The following results were observed when the raisins or figs were added to the basal ration.

1. A ration of 105 grams of raisins in bread produced fifty to sixty per cent as great an increase in the pH of the urine as did 300 grams of raisins. The increase produced by the 105 grams ranged from 0.45 to 0.60 pH units. Three hundred grams of figs produced an increase in the pH of the urine ranging from 0.9 to 1.1 pH units. This change was slightly greater than that produced by an equal weight of raisins.

2. A decrease in the ammonia excreted with a corresponding decrease in the total acidity was noted. The 300 grams of figs produced a greater decrease than 105 grams of raisins and also a slightly greater decrease than an equal weight (300 grams) of raisins.

3. There was an increase of the alkaline reserve, calculated according to the method of Fitz and Van Slyke (2), above the normal for each subject the greater increase being produced by the figs.

4. A correlation between the alkalinity of the ash and the physiological reaction was apparent. A more basic body reaction was associated with higher alkalinity of the ash.

5. The organic acids from 105 grams of raisins daily were completely oxidized by the body, whereas those from 300 grams were only 92.8 per cent oxidized. However, the organic acids from 300 grams of figs were oxidized to the extent of 97.5 per cent.

The writer wishes to express his appreciation of the interest and advice of Dr. W. V. Cruess, upon whose suggestion this study was initiated. The full cooperation of the men taking a part in the diets is gratefully acknowledged.

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GRAHAM LUSK, 1866-1932



## GRAHAM LUSK

### A BRIEF REVIEW OF HIS WORK

**F**OR the first time *THE JOURNAL OF NUTRITION* is obliged to report the death of one of its editorial staff. Professor Graham Lusk, who has been a member of the executive committee from the beginning and whose counsel has been sought since the Journal was first projected, died in New York City July 18, 1932. As a mark of the deep respect and high honor in which his memory is held, the editorial board and publisher place on record the following review of his life and work.

Graham Lusk was born in Bridgeport, Connecticut, February 15, 1866. His father was Dr. William Thompson Lusk, the distinguished obstetrician and author; his mother was Mary Hartwell Chittenden. He married in 1899 May W. Tiffany, daughter of the celebrated designer and manufacturer of art glass. Mrs. Lusk and the three children, William T., Louise (Mrs. Collier Platt), and Louis T., survive him.

His father desired that Graham should be a physician, but realizing that his impaired hearing would be a serious handicap, he advised that the next greatest service he could render to medicine would be as a physiological chemist. The father had been a physiologist for a time and he appreciated the important contributions chemistry was bound to play in the elucidation of the life processes. Accordingly, the boy was sent to the Columbia School of Mines for his foundational training in chemistry, where he was graduated in 1887. He then went to Germany for advanced education in physiology. Under Ludwig at Leipsic he learned the physical side of physiology and under Carl Voit at Munich the chemical side. Voit made by far the greater impression upon him and after receiving his Ph.D. at Munich in 1891 he returned full of enthusiasm for the views of the Munich school which just then were beginning to be known in America. Indeed it may truthfully be said that Lusk became the apostle to the Americans of the Voit-Rubner doctrines in nutrition.

Lusk's first academic position was that of instructor in physiology at Yale Medical School. He became assistant professor in 1892 and professor in 1895. Three years later he was called to the professorship of physiology at the recently reorganized University and Bellevue Hospital Medical College in New York City in which his father had been professor of obstetrics, a position which he held until 1909, when he was called to Cornell University Medical College to the chair made vacant by the retirement of Austin Flint. Professor Lusk had retired from this chair only a few weeks prior to his death.

For his dissertation at Munich Dr. Lusk published a paper on the influence of carbohydrate on the catabolism of protein, himself being the subject, which showed that the withdrawal of carbohydrate from the diet caused a larger destruction of protein in the body. From the Yale laboratory he published two papers on phlorhizin diabetes in the dog which became important reference points for this subject for the next 25 years. The constancy of the ratio of dextrose to nitrogen in the urine of dogs kept under the influence of the drug, was established, the great increase in protein metabolism, the absence of any influence of fat upon the ratio, and the fact that the dextrose from meat appears in the urine in advance of the nitrogen. There was also an important paper with W. H. Parker on the maximum production of hippuric acid in the rabbit, which showed that as much as 3 or 4 per cent of the protein catabolized in the body may be eliminated as glycine. It was suggested in this paper that glycine may be formed synthetically, a conception which was abundantly confirmed later by Magnus-Levy, A. I. Ringer, H. B. Lewis and others.

From the University and Bellevue physiological laboratory came not less than eight papers on phlorhizin diabetes (or *glycosuria* as Lusk later preferred to call this condition) in several of which he was assisted by P. G. Stiles, Arthur R. Mandel, and A. I. Ringer, and two important papers on diabetes mellitus. One of these, published with A. R. Mandel in the *Deutsches Archiv für klinische Medizin* advanced the conception of a "fatal" D:N ratio in the human disease, identical with the maximum ratio obtainable in the phlorhizinized dog. Other contributions of this period published by his pupils, but originated and inspired by Lusk were: one on the growth of suckling pigs fed on a skimmed milk diet by Margaret B. Wilson in which it was shown that growth is proportional to the total caloric intake; two by A. R. Mandel on the relation of the purin (alloxuric) bases to aseptic fevers; two by J. R. Murlin on the nutritive value of gelatin and one by A. I. Ringer on the influence of adrenalin in phlorhizin diabetes. Lusk's book, the "Science of Nutrition" made its first appearance in 1906. It undertook to interpret the early contributions of the Munich school of Carl Voit to American readers and to draw from more recent contributions by followers of that school and others the materials for a strong foundation of a new science. It had from the start a profound influence. This period of Professor Lusk's scientific career is fittingly concluded by his Harvey Society lecture on Metabolism in Diabetes, appearing in the 4th volume of the lectures 1908-1909. Lusk was the founder of this society and its first president.

The move to Cornell University Medical College, only one block distant

on First Avenue, in 1909 brought enlarged opportunities for prosecution of a program of research which had been forming in Professor Lusk's mind while he was revising his "Science of Nutrition." The second edition made its appearance coincidentally with this move to Cornell. During the summer of this year, while alterations for the laboratory at Cornell were in progress, Lusk went to Europe in order to put the finishing touches to his revision, and while there, on the recommendation of his first assistant who was working in the nutrition laboratory of F. G. Benedict at Boston, resolved upon the construction of a small respiration calorimeter of the Atwater-Rosa-Benedict type, suitable in size for study of the energy metabolism of dogs or of small children. What he desired most of all to investigate was the specific dynamic action of the amino acids. Dr. H. B. Williams, already a member of the department of physiology at Cornell, went to Boston and studied the construction of the calorimeter. J. A. Riche, trained by long experience in Benedict's laboratory, was engaged to operate the new calorimeter and assisted Williams in its construction, a large part of the mechanical work being done by these two men. Together with Professor Lusk they formed a research team of unusual ability, and the precision with which dependable results on this difficult problem were turned out was the result of clear comprehension of the physiological factors, combined with high technical skill. Williams, however, left soon to accept an appointment at the College of Physicians and Surgeons, Columbia University.

The first work in the order of publication accomplished by the calorimeter was a paper by John Howland on the energy metabolism of sleeping children. Lusk had very generously set aside his own program to give Howland this opportunity, which had much to do with making him professor of pediatrics at Washington University and, a year later, at Hopkins. This work at the same time demonstrated the remarkable efficiency of the calorimeter which Williams had built.

The first paper on the specific dynamic action of meat protein by Williams, Riche, and Lusk portrayed for the first time the hourly course of this phenomenon, particularly the high metabolism within the first few hours after ingestion (not at all disclosed by Rubner's work), critically examined the basis of calculation for the s.d.a., accounted for some discrepancies between the heat as measured and the heat calculated, and proved the retention of glycogen formed from the excess protein. He believed at this time that the cause of the dynamic effect was the stimulating action of amino acids on the protoplasm, causing more rapid oxidation. The first papers on the effect of carbohydrate also revealed a much higher dynamic



action in the early hours following ingestion than had been suspected from the results obtained by Rubner in 24-hr. experiments, but confirmed those found by Magnus-Levy in man.

The starting point of the program on specific dynamic action on the amino acids was Rubner's hypothesis that the extra heat is due to the metabolism of those fragments of the protein molecule not convertible into sugar. Lusk and Ringer had found that glycine and alanine yield all of their carbon as sugar when fed to the completely phlorhizinized dog, while glutamic acid yields only 3 out of 5 carbons. According to Rubner's idea glutamic acid should show the greater specific dynamic action in the normal dog. The opposite however proved to be the case. Glycine and alanine had a large effect, but glutamic acid none. He investigated the effect of two other amino acids, leucine and tyrosine, and found it intermediate between the two groups just mentioned. When all five were combined to produce a mixture approximately equal in nitrogen content to 100 grams of beef, the dynamic action was found to be greater than that of the meat because of the more rapid absorption. He argued strongly for the conception of mass action of the amino acids on the protoplasm, as opposed to the view of Rubner. When Miss Wishart found that there was no accumulation of amino acids in the tissue (muscles) after the ingestion of 1000 grams of meat, he gave up the idea of a direct stimulating effect of the amino acids. What seemed to him crucial evidence against the Rubner conception however, were: (1) the observation of Csonka that the sugar formed from glycocoll and alanine are eliminated just as rapidly in the phlorhizinized dog as is sugar administered as such, and yet (2) while the sugar elimination involved no extra energy production, glycocoll in isoglucogenetic quantity did so to the same extent as in the normal dog.

These facts indicated the possibility of a stimulating action from the hydroxy acids formed as intermediary stages in the metabolism of the amino acids. Long and involved experiments were therefore undertaken to compare the effects of keto with aldehyde sugars, ethyl esters of the hydroxy acids (ethyl lactate was the only successful one) with carbohydrate on the one hand and with ethyl alcohol on the other, and finally the ester with a combination of alcohol and sugar. The results seemed to show conclusively that the lactate stimulated far more than either alcohol or dextrose, and he adopted the view, held for several years, that the dynamic effect of amino acids is due to the stimulation produced by the hydroxy acids resulting from deamination.

Later experiments, however, shattered the foundations of this view and once more he promptly relinquished an explanation which had meant much

to him. Giving glycollic acid in the amount theoretically derivable from glycine, produced very much less heat—in fact seemed scarcely to raise the rate of oxidation at all. When he was able finally to secure satisfactory experiments with lactic acid, the same increase in production was obtained as from an equal weight of alanine. However, when lactic acid was administered to the dog together with sugar, it did not produce a summation of heat production as does an equal quantity of alanine. Lusk was obliged to conclude that the hydroxyacids derivable from the amino acids glycine and alanine do not explain the specific dynamic action of these substances. As a corollary to this work Miss Taistra and Chanutin independently proved that the alterations in  $\text{CO}_2$ -combining power of the blood resulting from ingestion of organic acids or of protein (or amino acids), respectively have no effect on the heat production and therefore cannot be invoked as playing any part in the mechanism of the specific dynamic action.

Professor Lusk now (1923) turned over experiments in his laboratory on the specific dynamic action of protein to Rapport, who alone and with Weiss, a medical research fellow from Prague, made some very interesting observations. They should be mentioned here because the experimental work was done directly under Lusk's guidance. Rapport found that six different proteins, when fed in such amounts as to contain the same quantity of nitrogen, gave substantially the same dynamic action, notwithstanding that they contained very different proportions of amino acids. Weiss and Rapport discovered that when a protein like casein or gelatin was fed in rather liberal amount together with either glycine or alanine the dynamic effect was the same as when the protein was fed alone. In other words, the specific effect of the amino acid was completely cancelled. Meat alone given in increasing amounts caused proportional increases in heat production, just as do increasing amounts of amino acids. Beef and casein also summated properly. The neutralizing effect of a protein (gelatin) on the dynamic action of an amino acid (glycine) was not due to alteration of absorption. Indeed the same neutralizing effect was found when the amino acid was given parenterally as when fed *per os*. The increase in metabolism after giving asparagine with glycine was not significantly different from that after giving glycine alone, showing that asparagine had no power to neutralize the dynamic action of glycine.

Two years later Plummer, Deuel and Lusk, starting from the observation of Weiss and Rapport just mentioned, compared the specific dynamic action of ingested glycyl-glycine with that of glycine on the hypothesis that the dipeptide might be absorbed as such and exhibit a lower effect

because of the peptide linkage. The experiments proved conclusively that such is not the case.

Also under Professor Lusk's guidance were the experiments of Nord and Deuel and of Gaebler designed to test the hypothesis that the adrenals or the hypophysis, respectively, may be concerned in the mechanism of the specific dynamic action of protein. The former proved that glycine given intravenously as well as orally to adrenalectomized dogs exhibited a dynamic effect comparable to its effect in (other) normal dogs. The latter showed that removal of the entire pituitary gland from a dog did not alter the specific dynamic action of meat in the second and third hours after ingestion. Incidentally Gaebler found that there was no parallelism between the concentration of amino acid nitrogen or total non-protein nitrogen in the blood and the increased heat production resulting from the ingestion of meat.

The last research on the specific dynamic action in which Professor Lusk participated directly was published in January, 1930, by W. H. Chambers and himself. It was an attempt to settle for glutamic acid the question which had been so decisively settled for glycine, with respect to the relative amount of the dynamic effect in the diabetic as contrasted with the normal organism. With glycine the effect was the same in the two—regardless of the fact that in the diabetic all the carbon of glycine was excreted as sugar. Since glutamic had no dynamic action in the normal dog, although three of the five carbons are excreted as sugar in the phlorhizinized dog, it was of interest to see whether this acid would exercise any dynamic effect in the diabetic animal. The experiment was as clear cut and final as had been the earlier one with glycine. Glutamic acid produced no increase in heat production, whether the animal was diabetic or normal. Meat however in like quantity produced the same amount of extra heat in both conditions.

What could explain these facts? Lusk had been obliged to abandon both his first and second theory regarding the dynamic action. He now read carefully the recent speculations of Aubel, of Meyerhof and of Adams on the possible explanation of these specific effects on the principles of thermochemistry and of thermodynamics. He was especially impressed by Adams' analysis indicating that the reaction, glutamic acid to glucose and urea, should take place spontaneously without the aid of outside energy, while the deamination of alanine and its transformation to sugar and urea would require approximately 60 Cal. per mol. of outside energy. The specific dynamic action then would arise from the necessity which the organism is under of effecting this transformation.

In his latest reviews on the subject of specific dynamic action Lusk very frankly adopts the original explanation of Rubner, that the increment of heat results from the metabolism of the intermediary products themselves. He says "the evidence accumulated since (1923) has tended to justify Rubner's general statement." He is referring to the thermochemical and thermodynamic considerations just recited. In his charming address on Rubner at Syracuse he says of him "Great men are very rare. They are worth knowing. They give impulse and stimulus to lesser men. They make the world more worth while for others to live in because of their presence in it. Max Rubner was the greatest man I ever knew."

The dynamic effect of carbohydrate and fat Lusk originally called the metabolism of plethora, or, as the writer has paraphrased this conception elsewhere, "oil on the fire." More fuel burns just because there is more of it available. But in the latest edition of his "Science of Nutrition" and in his recent reviews he states that on the evidence of others (notably of Mason, Baur, Carpenter and Fox, Dann and Chambers), this view must be revised; for all of these authors have shown that the specific dynamic action of glucose after its ingestion is not proportional to the amount of sugar oxidized (as judged by the respiratory quotient). Again the open mind—no pride of opinion—only the truth.

If Professor Lusk's long series of experiments and writings on the specific dynamic action illustrate his open-mindedness, his work and writings on diabetes equally well illustrate his adherence to ideas which he formulated early in his career. His experimental work on the disturbances to carbohydrate metabolism in the animal poisoned with phlorhizin, which had contributed so much to the understanding of the human disease diabetes mellitus, were subordinated for several years to the studies of specific dynamic action. But he was not able very long to refrain from further studies calculated to elucidate other aspects of this subject. In the Harvey lecture delivered in 1908 he had summarized the state of existing knowledge at that time in these statements.<sup>1</sup> "The requirement of energy for the maintenance of the life of a man is fixed and definite . . . The diabetic who cannot burn dextrose is thrown on protein and fat as sources of his potential energy . . . But it happens unfortunately that a major portion of the ingested protein is convertible into sugar in the diabetic organism, and . . . is carried away by the urine . . . To compensate for this, the protein metabolism increases, but fat metabolism remains the mainstay of life. . . . Conditions varying in severity also arise in which the end products of fat

<sup>1</sup> That these teachings are now so perfectly familiar is largely due to the influence of Lusk's writings.

metabolism, such as beta oxybutyric acid, aceto-acetic acid and acetone do not burn, but accumulate . . . and are eliminated in the urine." The sugar production from protein was definite in every known form of diabetes, though not necessarily the same in all.

From v. Noorden's clinic had come recently the idea that the endocrine glands were concerned in the variability of the D:N ratio, and indeed that the adrenal gland overpowering the pancreas might be *particeps criminis* in the causation of diabetes in man. Falta and his associates believed that epinephrin inhibited the internal secretion of the pancreas, thereby preventing oxidation of sugar and indirectly by exciting the thyroid, caused increased protein metabolism and production of sugar from fat. Ringer, as previously noted, had proved that epinephrin administered to a phlorhizinized dog, rendered free of glycogen by shivering, did not cause any increase in protein metabolism nor any increase in sugar production beyond the usual D:N ratio. Therefore the effects on protein and fat metabolism were disposed of. There remained the question of oxidation of sugar. The new calorimeter with its highly exact measurements of the respiratory metabolism in hourly periods afforded an opportunity of settling this question, and it was done in a few clean-cut experiments published in 1914. Epinephrin not only did not interfere with the oxidation of sugar, but because of its hypergycaemic effect actually increased it very sharply. The effect on total heat production was obscured by the restlessness of the animals.

An experiment published in brief form in 1913, together with his preliminary report of the above work on epinephrin, gave Lusk particular satisfaction because it was the result of a suggestion from Prof. v. Noorden who was in the country in 1912 in attendance at the International Congress of Hygiene and Demography at Washington. v. Noorden was shown an experiment done with the new calorimeter proving that the dog under phlorhizin intoxication exhibited an increased heat production comparable with that of the depancreatized dog. He remarked that if the thyroid were extirpated the increased heat production would be lacking, and so it proved to be. The specific dynamic action of protein food, however, was normal, and the high D:N ratios found after thyroidectomy and phlorhizin treatment were explained by excessive retention of glycogen, after the gland was extirpated but before phlorhizin acted. Thus he found additional evidence to assure him that his early position regarding the non-transformation of fat to sugar was correct.

The convincing evidence developed by Deuel and Milhorat in Lusk's laboratory, refuting the claim of Geelmuyden that acetic acid can be

transformed to sugar, also gave him great satisfaction; for it proved that even if acetic acid were an intermediary product of fat metabolism it would not constitute a link in the transformation to carbohydrate, but would be largely oxidized in the phlorhizinized animal. A further argument against this transformation in the dog which seemed to him irrefutable is given in his discussion of the specific dynamic action in the third volume of this journal. Should fatty acids of our common foodstuffs be so transformed before final combustion in the body there would result a net energy loss of 34 per cent according to Chauveau, or 21 per cent, according to the free-energy calculations of Borsook and Winnegarden. The dynamic action of fat ought therefore to be 21 per cent greater than that of the sugar into which it was transformed. Again, from his work with Anderson on the muscular efficiency of dogs, he found that carbohydrate was 5 per cent more economical than fat, but taking account of a lower basal metabolism induced by carbohydrate, as demonstrated by Dann and Chambers in his laboratory, the efficiency works out exactly the same for fat and carbohydrate. In other words the two non-nitrogenous food stuffs are mutually replaceable in isodynamic quantities in the support of muscular work. This could not possibly be true if fat had to be changed to carbohydrate before its combustion in the muscle took place.

Knoop once remarked to the writer that the thing he admired most in Graham Lusk was "the courage with which he stood up for his scientific beliefs." He never wavered on this subject of the transformation of fat to carbohydrate in the mammalian body after his original observation published with Reilly and Nolan in 1898 that fat does not increase the D:N ratio. He did concede to the writer in private conversation only a month before his death that it seems to be true in germinating oleaginous seeds.

A word is in order at this point concerning the writer's work on pancreatic diabetes which was in progress in Lusk's laboratory more or less continuously from 1912 to 1916. We were in search of the pancreatic hormone but failed of a consistent demonstration of its presence at this time. Professor Lusk was in no wise to blame for this failure. He approved the undertaking but was skeptical of any success for the treatment of diabetes along that line. Consequently he took very little interest in the experiments.

Meantime (1912) Lusk had secured the grant of money from the Russell Sage Institute of Pathology which made possible the construction of the calorimeter in Bellevue Hospital for the study of energy metabolism in disease. He selected E. F. DuBois as medical director and himself became scientific director of the program of studies. DuBois and Lusk worked harmoniously and enthusiastically together for twenty years. The results,

published in the long series of papers under the general title of Clinical Calorimetry, are too well known to require detailed review at this time. Only those parts of the program known to the present writer to have been of particular interest to "The Professor," as he was always affectionately called by the Sage group of workers, will be discussed here. Lusk himself wrote the first paper of the series setting forth the story of calorimetry to that time and describing the general principles of the Atwater-Rosa-Benedict type of calorimeter which was decided upon for their purposes. The second was a detailed description of its construction by Riche and Soderstrom who designed and built the calorimeter, the third and fourth contained descriptions of the metabolism ward in the hospital and of the first experiments on basal metabolism of the normal human subject done with the calorimeter, by Gephart and DuBois.

The fifth was the very significant paper by Delafield DuBois and E. F. DuBois on measurement of the surface area of man. In this enterprise Professor Lusk was extremely interested, for he believed in Rubner's law as in one of the eternal verities. In fact in the last public lecture which Professor Lusk gave (Syracuse, June 22, 1932) he spoke on the life and work of his friend Rubner who had died only two months before, and praised with particular emphasis Rubner's recent reiteration of his views on that subject. When the DuBoises hit upon the ingenious paper-mould method of measuring the surface and found the normal basal metabolism to be even more closely correlated with it than with surface as given by the old formula of Meeh, Professor Lusk's gratification was unbounded. There is no subject known to the writer in which he exhibited such intense—one might almost say passionate—interest. The broad truth of this law was too beautiful to be marred by slight exceptions here and there.

The sixth and seventh papers of the series were on typhoid fever by Coleman and DuBois. Then came a very clarifying paper by the professor upon the diabetic respiratory quotient. It discusses the manner in which the different components of the energy metabolism are related to each other, and how the diabetic condition affects this relationship.

All these papers appeared simultaneously in May 1915 and represented the first three years' results with the new resources. Simultaneously the papers on animal calorimetry in the medical school and clinical calorimetry in the hospital continued to flow from the two sides of First Avenue, for nearly twenty years. It is safe to say that together they constitute one of the most important assemblages of scientific results ever achieved under unified direction, in the related fields of physiology and internal medicine.

There are in the clinical calorimetry group seven other papers dealing

with the measurement of surface area and the basal metabolism of normal subjects of different ages by this standard. The DuBois formula and standards for different ages are now known and used the world over. It is certain that Lusk's critical eye scanned all these papers before publication. There are several also in which the specific dynamic action of foods was measured on human subjects, both normal and in diseased conditions, such as exophthalmic goitre, tuberculosis and diabetes, in dwarfs and a legless man, in all of which the professor was consulted daily, almost hourly. Several important papers have to do with the best experimental conditions for obtaining trustworthy results in which Lusk joined as author; a few others concerned with diabetes he helped write. But from about the 25th paper on his name no longer appears as author. He was now only consultant and sponsor. The papers continued to cover a broad range of subjects in the endeavor to bring scientific methods to bear upon the study of disease. The majority of the papers, not only in the calorimetry series, but those not involving the use of the calorimeter, are either directly concerned with the diseased subject or are directed toward the clarification of the pathological physiology encountered in disease. Here was the application of the German methods, for which Lusk had long contended; and when these methods became actually available for his own direction, jointly with men of clinical training, he was supremely happy.

This review of Lusk's work is by no means complete. Only the subjects in which he was most intensely interested have been mentioned. There were numerous other papers on medical education, on historical topics and characters, on subjects relating to nutrition in the war and many others which cannot be reviewed at this time.

Many honors came to Lusk in recognition of his high purposes and accomplishments in science. He was made Doctor of Science by Yale University, Doctor of Laws by the University of Glasgow, Fellow of the Royal Society of Edinburgh, Foreign member of the Royal Society of London, and member of the National Academy of Sciences. He served on important committees and boards during the war; for example, the advisory board of the Division of Food and Nutrition, Medical Department, U.S. Army, and the Interallied Scientific Food Commission. On the latter he, with Professor Chittenden, represented America and together they made a journey to England, France, and Italy in the most trying period of the war.

This paper should not close without a word concerning Professor Lusk's personal qualities. Others more competent and even closer to him than the writer recently have written beautifully and truthfully concerning his



character. At the summer meeting of the American Association for the Advancement of Science in June, it was the writer's privilege to introduce him as one of the evening lecturers. The following words occurred in the introduction. "For fourteen years your chairman was associated with the speaker as pupil, assistant, and colleague and he now states from the heart that he has never known a man who combined in so happy a way the solid merits of the scientist with all that is finest of courtesy, kindness, and culture in a true American gentleman."

J.R.M.

NOVEMBER, 1932

THE RELATION OF THE VITAMIN B COMPLEX TO  
RENAL ENLARGEMENT CAUSED BY CYSTINE  
AND PROTEIN IN THE DIET OF THE RAT\*

By

BERNARD B. LONGWELL, ROBERT M. HILL  
AND ROBERT C. LEWIS*(From the Department of Biochemistry, University of  
Colorado School of Medicine, Denver)*

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VOLUMINOUS work has been done in recent years on the role of protein and protein metabolites in the etiology of kidney disease. The possibility that the kidney might be injured if called upon to excrete the waste products of protein metabolism at a high level of concentration has occurred to numerous investigators. The findings of these investigators have been distinctly controversial in nature.

Newburgh (1) in 1919 was one of the first to attempt to throw light on this question. He found that rabbits' kidneys were injured by a high protein dietary. Squier and Newburgh (2) reported that the kidneys of human subjects were damaged by diets containing a high proportion of protein in the form of beef. Newburgh and Clarkson (3) observed kidney lesions in rabbits which had been subjected to diets containing 36.2 per cent and 26.8 per cent of protein, respectively. The intensity of the lesions appeared to be parallel to the concentration of the protein.

The results of the above work, most of which was done on rabbits, might be attributed to the fact that the animals are herbivorous and not accustomed to the ingestion of large amounts of protein. Polvogt, McCollum, and Simmonds (4) in 1923 reported work done on the rat, an omnivorous animal, that tends to refute such a contention. Using high protein diets, they noted lesions in the kidneys that involved the glomeruli and tubules. Osborne, Mendel, and Cannon (5) in 1924 failed to confirm these results on the rat. In their work the weight of the kidney was about twice that of

\*The experimental data in this paper are taken from the thesis submitted by Bernard B. Longwell to the Graduate School of the University of Colorado, August, 1931 in partial fulfillment of the requirements for the M. S. degree.

the kidney of the animal on a normal diet, but microscopic examination failed to disclose any abnormalities. Further work by Osborne, Mendel, Park, and Winternitz (6) substantiated these findings. Miller (7) also reported renal hypertrophy but no microscopic changes in rats' kidneys on high protein diets.

Confirmation of the earlier work was claimed by Evans and Risley (8) in 1925, who observed lesions in the kidneys of rats on diets high in protein content. Anderson (9), however, saw no lesions but did observe renal hypertrophy. Jackson and Riggs (10), and Smith and Möise (11) observed renal hypertrophy but no evidence of other damage on diets high in protein. MacKay, MacKay, and Addis (12, 13, 14, 15, 16, 17) have observed an increase in the size of the kidney as the amount of protein is increased in the diet or as cystine is added to the diet. On no occasion did they note any pathological changes in the kidneys when such diets were fed.

The question of whether renal lesions produced in this manner are due to the excretion of large amounts of the end products of protein metabolism, or to a toxic effect resulting from the failure of absorption products to be metabolized along their usual channels, has also been investigated. Newburgh and Marsh (18) in 1925 investigated the nephrotoxicity of several of the amino acids and found arginine, aspartic acid, histidine, lysine, tyrosine, tryptophane, and cystine to be injurious to the rabbit's kidneys. Curtis and Newburgh (19) and Lewis and Updegraff (20) also found cystine to be nephrotoxic.

Suggestion of a relationship between the amount of vitamin B in the diet and the effect of protein on the kidney was made by Drummond, Crowden, and Hill (21) in 1922. Reader and Drummond (22) also suggested such a relationship in the report of work which showed renal hypertrophy, but no histological changes, on high protein diets. Hartwell (23, 24) had definitely observed that an increase of protein in the diet necessitated that the vitamin B ration also be increased in order to enable the mother rat to raise a healthy litter. Reader and Drummond (25) again suggested such a relationship as a result of data obtained by them showing hypertrophy but no lesions of the kidney with diets high in protein but low in vitamin B. This condition disappeared when sufficient of the vitamin was fed. Still more convincing are the data of MacLean, Smith, and Urquhart (26) who were unable to produce signs of renal injury in rabbits with diets containing as much as 60 per cent protein as long as green food was given every day. When green food was withdrawn, renal injury developed as evidenced by albumin and casts in the urine. Further indication of such a relation-

ship was noted by Hassan and Drummond (27) and Hartwell (28), who observed that the protective factor resisted autoclaving.

Cox, Smythe, and Fishback (29) in 1929 observed definite tubular lesions in rats which had been subjected to diets containing 0.3, 0.6, and 0.9 per cent of cystine, respectively. This condition they diagnosed as "acute toxic nephrosis." Cox and Hudson (30) attempted to show a relation between the amount of vitamin B in the diet and this cystine nephrosis. They found that it was prevented by feeding 300 mg. of the Osborne and Wakeman yeast concentrate daily. They could find no relation between the development of nephrosis and the diet preceding the experimental period. They concluded also that the tendency to the development of the condition was an hereditary factor.

The controversial nature of the literature on this subject made it seem worthy of reinvestigation. The problem divides itself into three general questions. 1.—Do high concentrations of protein or cystine in the diet cause a definite pathological change in the kidney structure or, as some of the reports in the literature state, only an hypertrophy of that organ? 2.—Can the development of renal lesions or the hypertrophy, whichever occurs, be prevented or diminished in intensity by the use of whole yeast or yeast extract? 3.—What factor of the yeast extract is responsible for the beneficial effects, if any, obtained? The present paper is a report of an investigation of the first two questions.

#### EXPERIMENTAL

Male albino rats weighing between 30 and 65 grams at the beginning of the experiment were used. Such young rats are susceptible to cystine nephrosis, according to Cox and Hudson (30). The length of the experimental period was varied with the different groups as noted in the tables. The basal diet of Sherman and Spohn (31) was used and substitutions of cystine were made in this diet for the experimental groups.<sup>1</sup> Whole yeast extract was used as the source of the prophylactic agent. This extract was prepared by a method essentially the same as that described by Williams and Lewis (32).

The animals were kept in individual cages of the type used by Osborne and Mendel as described by Ferry (33). They were fed and weighed at regular intervals. Food and water were allowed *ad libitum*.

At the end of the experimental period the animals were weighed, killed

<sup>1</sup> In the preliminary work cystine was substituted for casein in the diet; in the experiments reported here in detail the cystine was substituted for corn starch.

by an occipital blow, and a complete autopsy was performed to ascertain if there was any gross abnormality that might be due to other causes than the effect of the diet. The kidneys were removed as quickly as possible, examined grossly, and all extraneous fat or other tissue was removed. The capsule was stripped off and the kidney sectioned longitudinally with a sharp instrument. A thin strip was made for tissue section, a cut being made on each side of the pelvis. This section was not more than 4 mm. thick and had a cut surface on each side so that fixation might take place as quickly as possible. Both kidneys were then immersed in a tared amount of Zenker's fixative in a glass stoppered bottle and weighed. The time that elapsed from the moment the kidneys were removed until they were placed in the fixing solution was never more than three minutes. Under these conditions post mortem autolysis was reduced to a minimum. After 12 to 24 hours fixation the kidneys were removed from the solution and sectioned for microscopic examination.

Comparison of the kidney weights was made with reference to the tables of Donaldson (34) and also by a study of the ratios between kidney weight and body weight. Hereafter, this ratio will be referred to as the K:B ratio.

### RESULTS

The results of kidney weight determinations for the first series of animals are given in Tables I and II. Comparison of Group I (basal diet) and Group II (basal diet and yeast extract), both on the basis of the K:B ratios and of the per cent deviation from normal kidney weights (Donaldson), shows that the vitamin B complex or some of its factors influences the development of the kidney. "Hypertrophy" of the kidneys appeared in those animals which did not receive yeast as a dietary supplement.

Groups III and IV, which received 0.3 per cent cystine, also show a marked difference in the size of the kidney in comparison to body weight. The kidneys of the animals of Group IV, which received whole yeast extract, were much smaller in comparison to body weight than those of Group III. Attention should be called to the fact that the comparative kidney sizes of Groups I and III are practically the same by both methods of calculation. This does not give to cystine at a level of 0.3 per cent in the diet any greater effect in the production of renal "hypertrophy" than the mere absence of the vitamin B complex from the diet.

Groups V and VI, which received a diet containing 0.6 per cent cystine, show a marked difference in comparative kidney weights. Seemingly, this is evidence that cystine at a level of 0.6 per cent in the diet does cause marked renal "hypertrophy" which is prevented by the addition of two

TABLE I  
EXPERIMENTS SHOWING THE EFFECT OF CYSTINE DIETS ON THE SIZE OF THE KIDNEY  
WITH AND WITHOUT THE VITAMIN B COMPLEX

Group	Rat No.	Experimental period (days)	Cystine (per cent) *	Yeast Extract *	Initial weight (gm.)	Final weight (gm.)	Kidney weight (gm.)	K:B ratio	Per cent deviation from normal (Donaldson)
I	37	21	0	0	35.0	36.0	0.626	0.017	+45.8
	38	21	0	0	34.1	40.7	0.720	0.018	+77.4
	39	21	0	0	36.2	44.1	0.632	0.014	+26.5
	40	21	0	0	35.8	35.0	0.527	0.015	+25.4
	Average				35.3	38.9	0.626	0.016	+43.0
II	41	21	0	2.0	24.1	66.2	0.800	0.012	+17.1
	42	21	0	2.0	34.1	76.4	0.891	0.012	+16.4
	43	21	0	2.0	30.0	82.7	0.908	0.011	+11.4
	44	21	0	2.0	34.0	80.3	0.838	0.010	+17.9
	Average				30.5	76.4	0.859	0.011	+15.7
III	45	23	0.3	0	51.7	51.3	0.676	0.013	+20.5
	46	23	0.3	0	50.8	55.6	0.860	0.016	+44.2
	47	21	0.3	0	43.7	31.5	0.663	0.021	+71.3
	48	23	0.3	0	48.5	37.4	0.781	0.021	+77.2
	49	23	0.3	0	46.1	54.4	0.687	0.013	+17.3
	Average				48.1	46.04	0.733	0.017	+46.1
IV	50	23	0.3	2.0	41.0	101.7	0.956	0.009	+ 5.7
	51	23	0.3	2.0	44.6	104.0	0.954	0.009	+ 6.1
	52	23	0.3	2.0	44.6	105.1	1.021	0.010	+ 6.3
	53	23	0.3	2.0	46.0	109.7	1.053	0.010	+ 2.8
	54	23	0.3	2.0	42.2	95.7	0.913	0.010	- 0.5
	Average				43.68	103.25	0.979	0.010	+ 4.1
V	55	22	0.6	0	41.6	30.0	0.663	0.019	+57.9
	56	22	0.6	0	40.5	35.5	0.706	0.020	+66.4
	57	22	0.6	0	36.8	37.4	0.708	0.019	+60.6
	58	22	0.6	0	44.5	36.8	0.711	0.019	+63.2
	59	22	0.6	0	41.7	29.1	0.754	0.026	+106.2
	Average				41.02	34.76	0.708	0.021	+70.8

\* In these diets the cystine replaced an equivalent amount of corn starch. Whole yeast extract was fed daily in a definite amount expressed in this table as gram yeast equivalents.

TABLE I—(Continued)

Group	Rat No.	Experimental period (days)	Cystine (per cent) *	Yeast Extract *	Initial weight (gm.)	Final weight (gm.)	Kidney weight (gm.)	K:B ratio	Per cent deviation from normal. (Donaldson)
VI	60	22	0.6	2.0	35.9	98.5	1.126	0.011	+20.0
	61	22	0.6	2.0	43.0	94.5	1.038	0.011	+14.6
	62	22	0.6	2.0	42.0	94.2	0.998	0.011	+10.3
	63	22	0.6	2.0	38.8	100.5	1.002	0.010	+ 5.1
	64	22	0.6	2.0	42.0	94.1	0.997	0.011	+10.3
<i>Average</i>					40.34	96.36	1.032	0.011	+12.1

TABLE II

EXPERIMENTS SHOWING THE EFFECT OF HIGH PROTEIN DIETS ON THE SIZE OF THE KIDNEY WITH AND WITHOUT THE VITAMIN B COMPLEX

Group	Rat No.	Experimental period (days)	Casein (per cent) *	Yeast Extract *	Initial weight (gm.)	Final weight (gm.)	Kidney weight (gm.)	K:B ratio	Per cent deviation from normal. (Donaldson)
VII	65	24	40.0	0	42.0	33.6	0.718	0.021	+76.8
	66	24	40.0	0	57.5	54.2	0.947	0.018	+62.2
	67	24	40.0	0	58.6	56.1	0.917	0.016	+52.7
	68	24	40.0	0	41.5	44.0	0.695	0.016	+39.2
	69	24	40.0	0	38.1	40.0	0.652	0.016	+40.5
	70	24	40.0	0	42.7	40.5	0.823	0.020	+75.6
<i>Average</i>					46.7	44.7	0.792	0.018	+57.8
VIII	71	24	40.0	2.0	64.7	125.0	1.500	0.012	+31.2
	72	24	40.0	2.0	50.0	121.5	1.236	0.010	+10.8
	73	24	40.0	2.0	60.0	136.7	1.492	0.011	+21.2
	74	24	40.0	2.0	61.3	138.8	1.473	0.011	+18.1
	75	24	40.0	2.0	44.0	112.8	1.171	0.010	+10.3
<i>Average</i>					56.0	126.9	1.374	0.011	+18.3

\* Whole yeast extract was fed daily in a definite amount expressed in this table as gram yeast equivalents.

gram yeast equivalents<sup>2</sup> per day of whole yeast extract prepared as noted above.

Groups VII and VIII (Table II), receiving 40.0 per cent casein in the diet, also show a marked difference in comparative renal weights. Group VII, which received no yeast extract, shows a 57.8 per cent increase over stated normals, a value which is not as high as that for Group V but distinctly higher than that of Groups I and III. Renal "hypertrophy" produced by a diet containing 40.0 per cent casein and prevented by the addition of whole yeast extract is indicated by these results.

It is worthy of note that the K:B ratio values for Groups II, IV, VI, and VIII, which received yeast extract, agree closely and are distinctly lower than the values for Groups I, III, V, and VII, which received no yeast extract. This demonstrates that the presence of the vitamin B complex, or some constituent of it, in the diet of the growing rat is necessary for the normal development of the kidney and that the kidney becomes larger when this necessary substance is lacking in the diet, the degree of "hypertrophy" depending upon other factors in the diet.

Microscopic examination of the kidneys of the animals which received casein showed normal renal structure in every instance. Of those which received cystine, rat 47 of Group III, on a diet containing 0.3 per cent cystine, developed a degenerative lesion in the tubular epithelium of both kidneys. The animal died two days before the end of the experimental period from this or some other undetermined condition. The kidneys of the remaining animals which received cystine were absolutely normal microscopically.

Table III gives the data for a second series of rats, all of whom received a diet containing 0.6 per cent of cystine. The dosage of yeast extract was varied in the different groups by increasing the yeast extract administration by 0.5 gram yeast equivalents in each successive group. This procedure was planned with the idea of determining the minimal dose of yeast extract which would be effective in overcoming the "hypertrophy" caused by 0.6 per cent cystine and to see if subminimal doses showed a graded response as the dosage was increased.

By comparison of both the K:B ratios and the per cent deviation from normal kidney weights (Donaldson), the results show that there was a graded response from Group IX (no yeast) to Group XII (1.5 gm. yeast equivalents). The kidneys of Groups XIII and XIV (2.0 and 2.5 gm. yeast

<sup>2</sup> The gram yeast equivalent is that amount of yeast extract which is obtained from the extraction of 1 gram of dry whole yeast. (Williams and Lewis, *Jour. Biol. Chem.*, 1930, 89, 275.)



TABLE III  
EXPERIMENTS SHOWING THE EFFECT OF VARIED AMOUNTS OF VITAMIN B COMPLEX  
IN OFFSETTING RENAL "HYPERTROPHY" PRODUCED BY 0.6 PER CENT  
CYSTINE IN THE DIET

Group	Rat No.	Experimental period (days)	Cystine (per cent) *	Yeast Extract *	Initial weight (gm.)	Final weight (gm.)	Kidney weight (gm.)	K:B ratio	Per cent deviation from normal. (Donaldson)
IX	76	30	0.6	0	39.5	51.0	0.752	0.015	+34.9
	77	30	0.6	0	47.5	75.0	0.835	0.011	+10.9
	78	30	0.6	0	48.7	52.5	0.700	0.013	+22.9
	79	30	0.6	0	51.5	61.8	0.902	0.015	+39.5
	80	30	0.6	0	53.0	58.0	0.657	0.011	+ 6.7
	Average				48.04	59.66	0.769	0.013	+22.98
X	81	30	0.6	0.5	48.0	64.5	0.821	0.013	+22.7
	82†	30	0.6	0.5	47.5	62.0	0.924	0.015	+42.5
	83	30	0.6	0.5	48.5	71.0	0.803	0.011	+11.3
	84	30	0.6	0.5	40.0	59.5	0.630	0.011	+ 0.3
	85	30	0.6	0.5	45.0	70.2	0.938	0.013	+31.1
	Average				45.8	65.44	0.823	0.013	+21.58
XI	86	31	0.6	1.0	39.5	76.5	0.710	0.009	- 7.3
	87	31	0.6	1.0	43.6	84.0	0.787	0.009	- 4.6
	88	31	0.6	1.0	40.8	76.5	0.793	0.010	+ 3.6
	89	31	0.6	1.0	37.8	72.5	0.789	0.011	+ 7.6
	90†	31	0.6	1.0	45.0	90.0	1.221	0.014	+40.1
	Average				41.34	79.9	0.860	0.011	+ 7.88
XII	91†	31	0.6	1.5	50.0	96.4	1.021	0.011	+10.7
	92	31	0.6	1.5	44.0	97.5	0.849	0.009	- 8.8
	93	31	0.6	1.5	48.5	102.5	0.948	0.009	- 2.2
	94	31	0.6	1.5	44.0	102.2	0.934	0.009	- 3.3
	95	31	0.6	1.5	49.0	96.8	1.059	0.011	+14.4
	Average				47.1	99.08	0.962	0.010	+ 2.16

\* In these diets the cystine replaced an equivalent amount of corn starch. Whole yeast extract was fed daily in a definite amount expressed in this table as gram yeast equivalents.

† Each of these animals had one fluid filled kidney. The weight of the kidney was taken after this retained fluid had been allowed to flow out.

TABLE III—(Continued)

Group	Rat No.	Experimental period (days)	Cystine (per cent) *	Yeast Extract *	Initial weight (gm.)	Final weight (gm.)	Kidney weight (gm.)	K:B ratio	Per cent deviation from normal (Donaldson)
XIII	96	32	0.6	2.0	41.4	94.5	0.996	0.011	+ 9.8
	97	32	0.6	2.0	44.5	116.0	0.990	0.009	— 7.8
	98	32	0.6	2.0	43.8	117.0	1.232	0.011	+13.8
	99	32	0.6	2.0	42.5	113.5	1.213	0.011	+15.1
	100	32	0.6	2.0	41.0	109.3	1.015	0.009	— 0.7
	<i>Average</i>				42.56	110.06	1.089	0.010	+ 6.04
XIV	101	32	0.6	2.5	43.2	116.3	1.128	0.010	+ 4.7
	102	32	0.6	2.5	40.5	91.0	0.967	0.011	+ 9.9
	104	32	0.6	2.5	47.0	108.9	1.068	0.010	+ 4.9
	105†	32	0.6	2.5	44.3	121.6	1.338	0.011	+19.8
	<i>Average</i>				43.75	109.7	1.125	0.011	+ 7.86

equivalents, respectively) show a slight increase above Group XII when compared to the Donaldson standards but very little difference in the K:B ratios. Evidently the minimal dose of the whole yeast extract used in this work that is necessary to prevent kidney "hypertrophy" when the diet contains a 0.6 per cent concentration of cystine lies somewhere near 1.5 gram yeast equivalents per rat per day.

Periodic examinations of the urine were made on the animals of these last six groups. The rats were placed in metabolism cages and the urine was collected under toluene at approximately 24 hour periods. At no time were there any casts, pus cells, erythrocytes, or amorphous sediment in the urine. There was at all times a slight albuminuria which did not increase during the course of the experiment. This was no more than is normal for the male rat.

Examination of the kidneys of these animals at autopsy revealed a gross abnormality resembling hydronephrosis in one kidney of each of four animals of the twenty-nine examined. Rat 82 had the right kidney involved and rats 90, 91, and 105 showed the condition in the left kidney. In each case the kidney contained a large quantity of yellow fluid which distended the organ to twice its normal size. Microscopic examination of the involved kidney showed that the medullary tissue had undergone pressure atrophy due to the presence of the retained fluid and, as a consequence,

was much thinner than normal. However, the remaining medullary tissue and the cortical tissue, as well as the tissue of the uninvolved kidneys, were entirely normal in appearance. Examination of the kidneys of the other twenty-five animals in these six groups revealed no abnormalities.

### DISCUSSION

Our experimental data show that both cystine (at a level of 0.6 per cent of the diet) and casein (at a level of 40 per cent of the diet) cause an enlargement of the kidney when whole yeast extract is not given as a dietary supplement. The administration of whole yeast extract prevented such an enlargement. Graded doses of yeast extract appear to have a graded action upon the size of the kidney as compared to body weight. This gives to some component of yeast, probably vitamin B complex or one of its factors, the property of preventing the increase in size resulting from the action of cystine or excess casein in the diet. These findings agree with the reports in the literature previously cited (25, 26, 27, 28, 30).

Quotation marks have been used advisedly in this report when speaking of "hypertrophy." Whether it is a true, definite, organ hypertrophy, which would persist if the causative factors were removed, or whether it is merely a work enlargement, which would disappear with removal of the cause, was not determined.

Notable throughout this work is the *complete absence* of evidence that a diet high in protein or cystine produces pathological lesions in the kidneys. Regardless of the type of diet, there was no evidence of degenerative kidney lesions with the single exception noted above (rat 47, Group III). Since this condition developed in this one individual only, it is improbable that it was due to the cystine in the diet. Our failure to produce kidney lesions is contrary to the observations of Cox, Smythe, and Fishback (29) that "cystine nephrosis" develops in rats fed cystine during a short experimental period. In our experiments with casein, a 40 per cent concentration of this protein was in no way nephrotoxic. It should be noted that our experimental period was probably much too short for the development of renal lesions, since Newburgh and Johnston (35) have reported that many months on a high casein diet are required to produce pathological changes in the kidney.

Further evidence that cystine is not nephrotoxic is given by the results obtained in some preliminary work done before the experiments reported above. Rats were given the basal diet used throughout our work plus 0.3 per cent cystine, an amount which had been found by Cox, Smythe, and Fishback (29) to be nephrotoxic to rats. The animals used were from a local

supply source and were in poor nutritive condition when received. Their general state of health was so poor throughout the experimental period that no attempt was made to draw conclusions from the data obtained concerning the effect of the diet on kidney weight. However, it is interesting to note that microscopic examination of the kidney tissue gave no evidence of pathological changes. Positive pathological findings in this group of animals would have had no weight, since animals in a poor nutritive condition might be expected to have a greater tendency than more healthy animals for the development of pathological lesions. The negative findings which were observed, however, seem most significant.

As stated previously, this work was begun with the intention of identifying the factor present in the water soluble portion of yeast that is responsible for its activity in the prevention of cystine nephrosis. The consistently negative tissue findings encountered in the early part of the work necessitated the development of the problem along altogether unexpected lines. Further work on the identification of the factor or factors of yeast extract responsible for the prevention of renal hypertrophy caused by cystine is now in progress in this laboratory and will be reported at an early date.

#### CONCLUSIONS

1. Vitamin B complex in the diet prevents abnormal development of the kidney of the rat under the influence of cystine or excess casein.

2. Cystine, 0.3 per cent, in the diet does not cause renal "hypertrophy" to any greater degree than the mere absence of yeast extract from the otherwise normal diet. A concentration of 0.6 per cent cystine, however, does cause an "hypertrophy" which may be prevented by the administration of vitamin B complex.

3. Excess casein in the diet likewise causes renal "hypertrophy" which is also prevented by the administration of vitamin B complex.

4. Graded doses of whole yeast extract cause a progressively decreasing response on the part of the kidney to the administration of cystine. This is true up to a certain point in the vitamin dosage. Increase above that point does not have any further beneficial effect.

5. Cystine was *not nephrotoxic* to these animals in the sense of causing degenerative lesions of the kidney.

The tissue sections used in this work were examined by Dr. W. C. Johnson, Head of the Department of Pathology, who confirmed our findings. The authors wish to express thanks to Dr. Johnson for his kindly advice and suggestions during the course of this problem and to the technical

staff of his department for their help in the preparation of tissues for microscopic examination.

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# THE METABOLISM IN PREGNANCY

## IX. THE FOETAL INFLUENCE ON THE BASAL RATE\*

By

ALLAN WINTER ROWE AND WILLIAM CLOUSER BOYD

(From *Evans Memorial, Massachusetts Memorial Hospitals, Boston*)

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IN A SERIES of earlier papers the senior author and his associates (1) have discussed the results of extended investigation on certain aspects of the metabolism in normal pregnancy. Subjects for this study were drawn both from an out-patient prenatal service (Series "A") and from two maternity institutions (Series "B" and "C") in order to secure a group truly representative of the conditions of practice. All studies were continuous from the time of initial contact through the completion of the pregnancy and for a number of weeks beyond, an experimental condition that added materially to the investigative difficulties but increased proportionally the significance of the data secured. Only those patients were retained who demonstrated complete normalcy of function throughout the period of observation. As all of the descriptive data have already been presented in the earlier papers, only a few of the fundamental records need be repeated here for purposes of orientation.

TABLE I  
FUNDAMENTAL DATA

Datum	Series			Total or average
	A	B	C	
Number of cases	25	21	31	77
Age, (years)	28	18	19	22
Height, (cm.)	158.8	160.4	160.2	159.8
Weight, † (kg.)	67.0	64.4	66.8	66.3
Weight increase, kg. per week	+0.32	+0.52	+0.54	+0.46
Weight of baby, (kg.)	3.31	3.20	3.28	3.27
Interval of study				
Maximum	34 weeks	13 weeks	25 weeks	
Average	21 "	8 "	14 "	

† 1 week ante-partum.

\* Presented before the American Physiological Society, Montreal, April 1931.

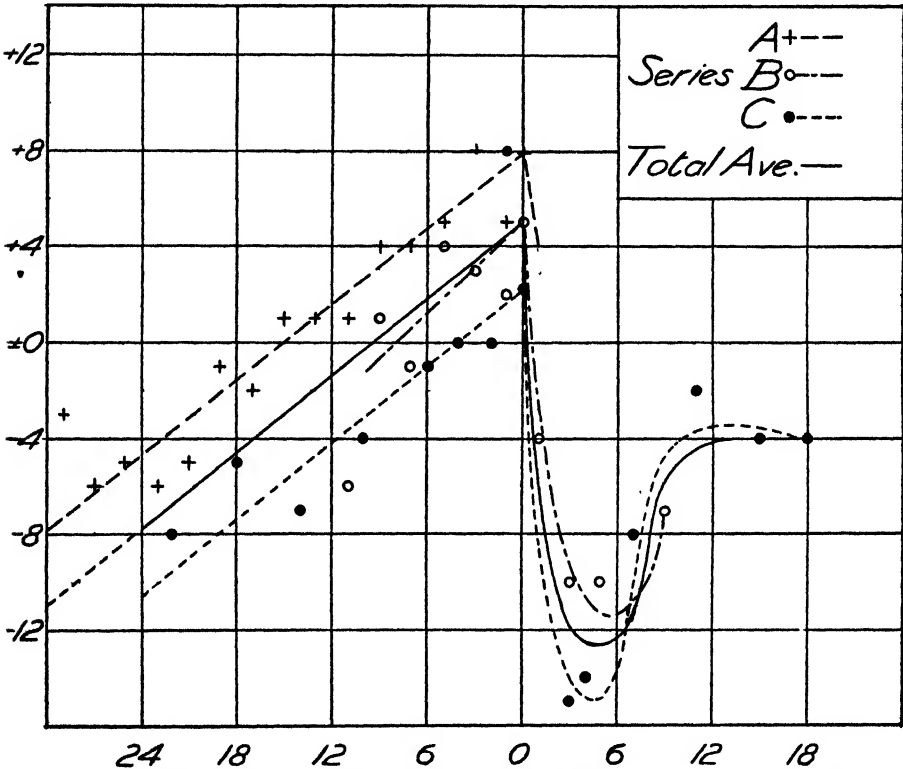
But minor comment is necessary. The youth of the group was determined largely by the sources of Series "B" and "C." Comparison of the data of these series with those of an older group studied in another connection and not included here shows that the present figures are wholly representative. The weights are given one week ante-partum as there is some evidence to support the contention that a loss of weight occurs in the few days immediately before delivery. The point is not wholly established, however, and the present convention avoids uncertainty.

The interval of study was naturally determined by the time of initial contact. In Series "B," admissions to the home were usually in the later months of gestation and the duration of the study was correspondingly circumscribed. The average duration in both Series "A" and "C" is unduly affected by the inclusion, for other purposes of the whole investigation, of a few cases who were first seen as the pregnancy was approaching termination. Adequate data are available to construct wholly representative curves for Series "A" and "C" over intervals of 27 and 21 weeks respectively.

A highly interesting and important datum is found in the estimation of the energy requirement as measured by the respiratory exchange (1, b, f). The results can be presented most compactly in graphic form. To absorb the differences produced by changing weights the deviations from prediction rather than the actual observed rates are plotted, thus indicating the relative changes. Following the practice of this institution, comparisons are made with the standards both of Harris and Benedict (2) and Aub and DuBois (3) and the deviation from their mean recorded. With the exception of Series "B", where the difference approximates 6 per cent, the variation between the predictions by the two formulas is 3 per cent or less.

The curves from the three independent series give entirely consistent results, each showing a linear upward change during the progress of the pregnancy; the slopes are substantially identical—exactly so in the case of the two longer and thus more reliable periods of study. The absolute difference of 6 per cent between these two extreme curves is readily explained by the varying conditions of testing. Series "A" was drawn from an out-patient service and the subjects came from their homes to the hospital on the morning of the measurement. While every effort was made to insure basal conditions before testing by the usual resting procedure, these subjects were patently less basal than were the patients constituting Series "C." With these latter, all of whom were hospital inmates, the machine was carried to the bedside and in many instances the patient was aroused

from the night's sleep in order to apply the test. The difference in the approach to basal conditions of the two groups is reflected in the observed rates. The solid line gives the average of the entire group and may be regarded as a representative portrayal of the changes occurring in pregnancy. It will be noted that the point of origin of each curve is below prediction and that the level at the time of delivery is superior to the cal-

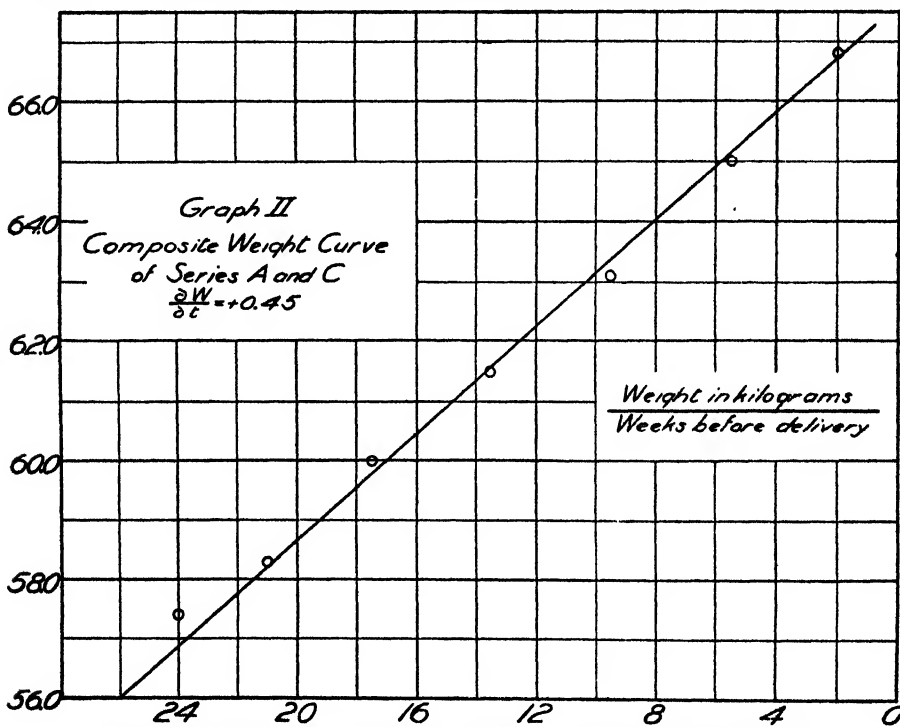


GRAPH 1. Variations of observed from predicted Total Basal Metabolism for 24 hours during the last six lunar months of pregnancy. Prediction standard is the average of the Harris-Benedict (2) and Aub-DuBois (3) indications.

culated normal. This point will be discussed later in the body of the paper. Patently, during the last 24 weeks of gestation at least, there is a progressive increase in the total basal for 24 hours of the order of 13 per cent over and above that conditioned by the increase in weight. The pregnancy is the major factor of difference between these patients and a like series of women demonstrating a progressive obesity but in a state of sexual rest and to this special physiological condition one turns for an explanation of the increased energy requirement.



Some years ago, Sandiford and Wheeler (4) analyzed the data of a single patient studied throughout gestation. They reached the conclusion that the foetal metabolism, as correlated with the surface changes engendered by foetal growth, was adequate to account for the entire difference. More recently Boothby and Sandiford (5) have made a second study on the same patient in a later pregnancy and again have reached the same conclusion. The study of a single case makes no allowance for the variations



GRAPH 2. Linear increase in weight during last six lunar months of pregnancy.

recognized to occur normally in this magnitude—the conventional allowance of  $\pm 10$  per cent in defining the zone of normal performance is an illustration. With so large a series as that under consideration and with the undoubted correlations between the component groups, it seems warrantable to assume that these present data may serve as the basis for a rigorous investigation of the validity of the conclusion given above. The present paper deals with such a survey. Studies of the individuals composing this group show that the maternal increase in weight, at least for the last 6 lunar months, is substantially linear (1, i). A composite of those cases in Series "A" and "C"—a significant majority—where the period of study was of adequate length may be used for illustration.

From the basic biometric magnitudes and the curves of deviation of the basal rates it is easy to calculate both the actual rates—from which the curves were initially derived—and those rates predicted by the two accepted standards. In this way, the actual deviations in terms of calories per 24 hours can be readily computed. The data are collected in the next table.

TABLE II  
BASAL RATE VARIATIONS. (CALORIES)

Weeks Ante-partum	Observed Rates from Curves				Deviation from Prediction			
	A	B	C	Com- bined*	A	B	C	Com- bined*
27	1315				- 84			
25	1336			1272	- 69			-112
23	1358			1296	- 54			- 97
21	1379		1292	1320	- 39		-128	- 82
19	1400		1317	1344	- 24		-113	- 67
17	1422		1343	1368	- 9		- 98	- 52
15	1443		1369	1393	+ 6		- 83	- 37
13	1464		1395	1417	+ 21		- 68	- 22
11	1485		1420	1440	+ 36		- 53	- 7
9	1506	1477	1446	1464	+ 51	- 7	- 38	+ 8
7	1527	1506	1471	1488	+ 66	+ 11	- 23	+ 23
5	1548	1534	1497	1513	+ 81	+ 29	- 18	+ 38
3	1570	1564	1522	1537	+ 96	+ 48	+ 7	+ 53
1	1591	1593	1548	1560	+111	+ 67	+ 22	+ 68

\* Calculated from the solid line in Graph 1 which represents the average of the entire study group.

In the table above the results under the caption "combined" are drawn from the average of the data of the complete group. Inspection shows that there is an increase in the energy requirement of between 7 and 8 calories per week in excess of that conditioned by the increment of the maternal weight. Series "B" shows a somewhat higher value but in this group the period of ante-partum study was relatively brief and thus the data lack the authority of those from the more protracted studies.

Experience has demonstrated that the surface area of the living organism is a biometric unit of great convenience as a standard of reference and the area, calculated from height and weight, is a fundamental factor in the widely used Aub-DuBois prediction formula.

Using the well-known Lissauer (7) formula,  $S = 10.3W^{0.667}$ , Sandiford (6) calculated foetal areas and these were used as the basis of calculation in

the two papers (4, 5) already mentioned. In a long series of biometric studies by Scammon and his associates, one publication by Klein and Scammon (8) gives the results of a series of actual measurements of foetal surface and derives formulae based upon these observations for the calculation of the area from the observed crown-rump and crown-heel heights and from the observed weight. In a later paper the same authors (9) present additional evidence of the applicability of these formulae. Scammon's measurements were made upon the dead foetus and he with Calkins (10) had previously noted a difference of 220 gms. between the weight of the live child and that of the dead foetus. Desiccation with loss of weight obviously takes place, a fact which was quantitatively recorded by Ipsen (11) in 1894. In Scammon's table the weight at birth is recorded as 3.00 kgm. which would correspond to a weight of 3.22 kgm. for the living child. This value, fortuitously, is practically the same as the averages of our several series (see Table I). Loss of weight by desiccation will entail a diminution of surface area so that a correction must be made for weight differences if living areas are to be calculated by the Scammon weight formula,  $S = 5.188 W^{0.75}$ . We have taken Scammon's actual weight records and computed the foetal areas at different periods of development by use of the formula given above and then made suitable correction for the weight differences between our own mean birth weights and that of Klein and Scammon,<sup>1</sup> (Columns 3, 4, 5, 6 of Table III). Further, we have calculated the foetal weights from our surfaces by the Klein and Scammon weight-area relation (based upon actual measurement) and to these values have applied the Lissauer surface formula (Column 7, Table III). Finally, we have reviewed the surface areas computed by this formula by Boothby and Sandiford (5) from weights which they derived by computation (Column 8, Table III) and have also performed a similar review of the values recorded by Sandiford and Wheeler (4) (Column 9, Table III). In this last series (Column 9) the final weight was estimated by the authors as 3.90 kgm. and the surface computations were based by them upon this with the proper decrements for the preceding time intervals. The actual weight of the child as delivered was 3.60 kgm. and the recorded surface values are consequently somewhat too high. The results of the several series of computations are collected in the following table.

The agreement of the results in our own several series is to be anticipated as the uniformity of the data from which they are derived ensures this

<sup>1</sup> The Scammon equation is written  $\text{Surface} = 5.188 (\text{Weight})^{0.75}$ . Our weight can be taken as  $R \times W$  where  $R$  = the ratio between live and dead weights. The maximum differences introduced by this correction are of the order of 8 per cent or less.

TABLE III  
FOETAL AREAS. (square meters)

1	2	3	4	5	6	7	8	9
Weeks	Scammon & Klein original data	Modified Scammon Formula (R & B Data)				Lissauer Surface from		
		Ser. A		Ser. B	Ser. C	Average	Wt. (Scammon)	Wt. (B&S)
		Ser. A	Ser. B	Ser. C	Ser. C			
28	0.004	0.004	—	—	—	0.004	—	—
24	.015	.016	—	0.016	—	.016	0.021	0.040
20	.034	.036	—	.036	—	.036	.042	.050
16	.059	.064	—	.063	—	.063	.071	.080
12	.090	.097	0.62	.096	—	.096	.101	—
8	.126	.136	.133	.135	—	.135	.136	.150
4	.166	.179	.175	.178	—	.178	.174	.200
0	.210	.226	.221	.224	—	.224	.214	.260
Child (Weight)	3.00	3.31	3.20	3.28	3.27	3.00	3.30	3.90*

\* Estimated by Sandiford & Wheeler and used in the computations. Actual weight = 3.60 kg.

result. Equally, the agreement with Scammon's actual observations is very close and for the same reason this was to be anticipated as we use his formula based on his own actual measurements. Less good, certainly in the earlier months, is the agreement with the Boothby and Sandiford calculations. At these periods, however, the foetus is very small and the addition which it makes a scarcely significant one (*v.i.*). As noted above, the Sandiford-Wheeler figures are too high and correction here would improve the correlation with the results from the Scammon formula but still leave the agreement poor in the earlier months.

The maternal areas at the several stages of gestation can be readily calculated from the maternal weights by means of the formula of DuBois and DuBois (12). We are inclined to question the convention adopted in the two papers under discussion, of subtracting the foetal from the maternal weight before computing the latter area. Certainly the increase in the pelvic and abdominal contents produces a definite increment in the surface area of the mother. The area calculated from total weight for the sake of equality will be used as the basis of the subsequent comparisons. This naturally will give slightly higher values for the maternal area than those calculated in the Boothby-Sandiford-Wheeler studies. In any case the difference is a minor one (a maximum of less than 3 per cent) and does not materially affect the final result of the next correlation.

The method of approach in the two papers under analysis was to calculate the maternal and foetal areas independently and combine them to express the area of the pregnant organism. In other words, by adding the foetal to the maternal surface, the total effective area is obtained and the heat production is referred to this as the biometric standard of comparison. The warrant for this approach may be questioned—and is by the present authors—as it is difficult to see how a selective heat loss could take place from a body under the presumably adiabatic conditions that obtain for the foetus in pregnancy. The foetal heat production, as we conceive it, would depend on the active mass of protoplasm—of which the surface is not an exact measure—and would ultimately be lost by conduction through and radiation from the maternal organism as is the energy from the viscera. The present thesis is to analyze the above assumption from the standpoint of its consistency with the actual data of observation.

Reverting to the exposition, by dividing what we may term the total effective area into the total caloric output for 24 hours, and dividing this result in turn by 24, the heat production per square meter of "effective area" per hour is secured. The results of this manipulation are given in the next table. To avoid an unnecessary multiplication of concordant figures,

comparisons are reported only with the data of Series "A" to serve as illustration and with those which are the average of the entire group (Referred to subsequently as "Total").

There is an approximate constancy to this final quantity, the range in the "A" series being 0.98 in 34 to 35 calories, while the combined group shows the slightly smaller and non-significant total variation of 0.78 calories in 33 to 34, a matter of less than 3 per cent. Recalculating the maternal complement by using the Boothby-Sandiford-Wheeler convention of de-

TABLE IV  
HEAT PRODUCTION IN TERMS OF TOTAL EFFECTIVE SURFACE

Weeks	Area Mother and Foetus		Obs. B.M.R.		Heat Prod. per sq. meter	
	A	Total	A	Total	A *	Total
27	1.604	—	1315	—	34.16	—
25	1.617	1.577	1336	1272	34.43	33.61
23	1.633	1.596	1358	1296	34.65	33.84
21	1.650	1.617	1379	1320	34.82	34.01
19	1.669	1.640	1400	1344	34.95	34.15
17	1.690	1.663	1422	1368	35.06	34.27
15	1.712	1.689	1443	1393	35.12	34.39
13	1.736	1.717	1464	1417	35.14	34.39
11	1.761	1.745	1485	1440	35.14	34.38
9	1.787	1.775	1506	1464	35.11	34.36
7	1.814	1.807	1527	1488	35.07	34.31
5	1.843	1.838	1548	1513	35.00	34.30
3	1.873	1.870	1570	1537	34.93	34.25
1	1.904	1.903	1591	1560	34.82	34.15

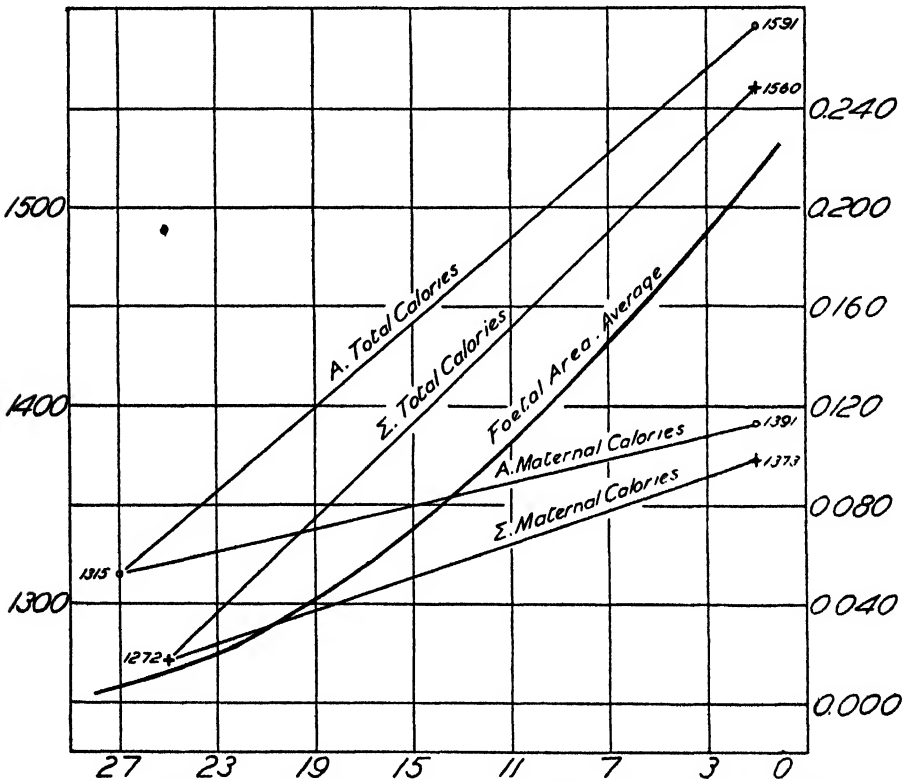
ducting the foetal from the total weight, the result is practically the same, 1.16 in from 33.5 to 34.5 calories. These figures, at first sight, certainly seem to confirm the conclusions reached in the two papers under discussion. It will be noted, however, that the values of the heat production per square meter show a definite progression defining a curvilinear relationship with a maximum approximately midway in the series.

A more rigorous analysis of the existing data can be secured through determining the excess heat production due to the foetus by deducting the maternal from the total caloric production and then comparing these values with the foetal area.<sup>2</sup> The underlying approach can be shown

<sup>2</sup> In calculating the maternal heat production, the assumption is made that at some selected time before delivery the foetal influence on the energy exchange, at first of necessity inconsiderable, makes itself manifest in tangible amount (See Graph V). From this point on, the maternal prediction is based on the total weight increases demonstrated by actual measurement. This

graphically by plotting the total and maternal output. The intercepted area is that which is ascribed presumably to the excess foetal production.

All of the existing data, both in these series and in the few others recorded in the literature, show a linear change in the relative basal rate. Equally, all of the computations of foetal area (see Table III) define a curvilinear relationship. Naturally any arithmetical adjustment of these



GRAPH 3. Total and maternal heat production with synchronous change in Foetal area. Curves marked "A" from Series "A," marked  $\Sigma$  from the "Total" group.

two quantities must determine a curvilinear change. The magnitude of the variation can best be demonstrated by the figures themselves. They have been computed for the several groups and the results for Series "A" and the "Total" group are assembled in the next table.

The variation here is of a very significant magnitude and certainly does

assumes that the total protoplasmic mass (based on the total weight) is related to the energy exchange of the pregnant woman as experience has shown it to be in the non-pregnant. The weight is the sole variable in both of the prediction formulae under present conditions. The warrant for this convention is discussed later in the text.

not imply any constant heat production by the foetus. Further, selecting any of the individual values of the foetal heat production and calculating the area changes necessary to give constancy to the output, a surface curve is defined that bears no relation to any of those based upon actual measurement as given in Table III. To illustrate, if the value for the foetal area, 0.106 at 11 weeks ante-partum in Series "A," be assumed to be correct, then the area at 21 weeks should be 0.40 and at one week 0.171. Any such attempted adjustment would but ill accord with facts and yield no more than an arithmetical artifact.

The method followed in calculating the maternal heat production from the gross measured maternal weight includes the weight of the foetus as well. The legitimacy of this procedure may well be questioned as it thus includes the weight prediction factor of the foetus when the Harris-Benedict formula is used. This gives too high a value for the maternal energy requirement and consequently, by difference, too small an amount for the foetal heat. The same objection does not hold when the Aub-DuBois generalization is used as there is an actual increase in the external surface of the mother as the uterus enlarges.

In order to estimate the order of magnitude of the error deriving from the inclusion of the foetal weight in predicting by the Harris-Benedict formula, the maternal contribution has been calculated after deduction of the weight of the foetus. The values thus obtained have been averaged with the uncorrected Aub-DuBois prediction. Further, that the story might be complete, the Aub-DuBois areas have been corrected by subtracting the foetal from the maternal weight before calculating the area, and the mean of the corrected<sup>3</sup> Aub-DuBois and corrected Harris-Benedict predictions compared with the values actually obtained. The results can be presented most compactly in graphic form.

At first sight, the greatest constancy would seem to obtain with the doubly corrected values. Even here, however, omitting the value at 23 weeks, the difference is 8.0 calories as against 10.2 for the wholly, and 8.8 for the partly, corrected predictions. Further, arbitrary manipulation of the foetal caloric output does not improve the situation unless the maternal contribution be assumed to follow a curvilinear course which compensates for that of the surface variation. For such arbitrary correction there is no experimental support. One other point to be noted is that with each deletion of the maternal heat production, that calculated for the foetus

<sup>3</sup> This is an over correction as it omits the actual increment in the maternal area deriving from the increase in the pelvic contents (*v.s.*).

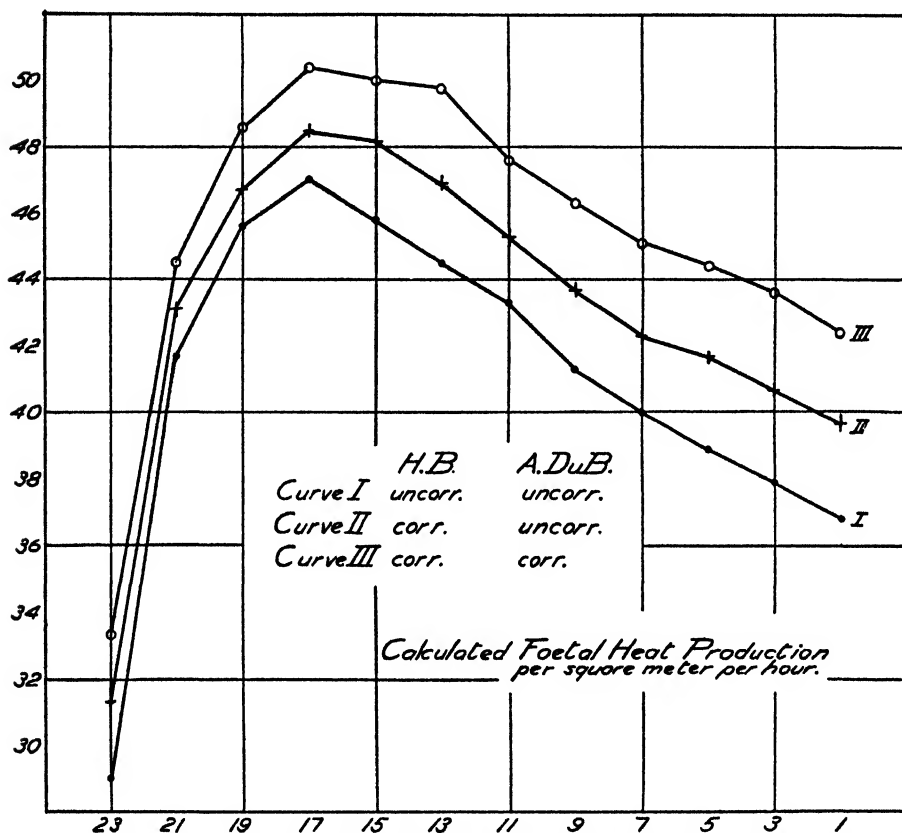


TABLE V  
FOETAL HEAT PRODUCTION

Weeks	Total Cal. (obs.)		Maternal Cal.		Foetal Cal.		Foetal Area		Foetal Cal. per Sq. Meter per hour	
	A	"Total"	A	"Total"	A	"Total"	A	"Total"	A	"Total"
27	1315	—	1315	—	—	—	0.006	—	—	—
25	1336	1272	1320	1272	16	—	.012	0.012	55.56	—
23	1358	1296	1326	1282	32	14	.020	.020	66.67	29.17
21	1379	1320	1332	1290	47	30	.030	.030	65.28	41.67
19	1400	1344	1338	1298	62	46	.042	.042	61.51	45.64
17	1422	1368	1344	1306	78	62	.056	.055	58.04	46.97
15	1443	1393	1349	1316	94	77	.071	.070	55.17	45.83
13	1464	1417	1355	1324	109	93	.088	.087	51.61	44.54
11	1485	1440	1361	1331	124	109	.106	.105	48.74	43.25
9	1506	1464	1367	1340	139	124	.126	.125	45.97	41.33
7	1527	1488	1373	1348	154	140	.147	.146	43.65	39.95
5	1548	1513	1379	1357	169	156	.168	.167	41.92	38.92
3	1570	1537	1385	1365	185	172	.191	.189	40.36	37.92
1	1591	1560	1391	1373	200	187	.214	.212	38.94	36.75

lies progressively farther away from the actual amount determined by experiment both in the mother and in the new-born child.

The basal metabolism is potentially a measure of the active protoplasmic mass and body weight is a rough measure of this quantity even though it can be no more than a first approximation because of the di-



GRAPH 4. Comparison of calculated foetal heat production per square meter per hour, based upon application of correction of foetal weights to value of maternal weight used in computing maternal energy alone.

versity of the tissues involved. Calculating foetal weights by the Scammon equations, the heat production per kilogram per 24 hours is readily computed.

As partly explained above, three methods of calculation have been used. All assume a relative constancy in the maternal contribution from some point in the pregnancy as illustrated in the data of Table V. The first calculation approach (I) uses the gross maternal weight (observed) as the "W" in both the Harris-Benedict and Aub-DuBois prediction equations.

The second (II) deducts the foetal weight from the gross maternal weight to yield the arithmetical value for the "W" of Harris-Benedict but persists in the first convention as regards the Aub-DuBois "W." The third (III) and last uses this modified "W" (gross maternal minus foetal weight) for the area computation of the Aub-DuBois prediction as well as the modified Harris-Benedict value which appeared in the preceding (II) computation.

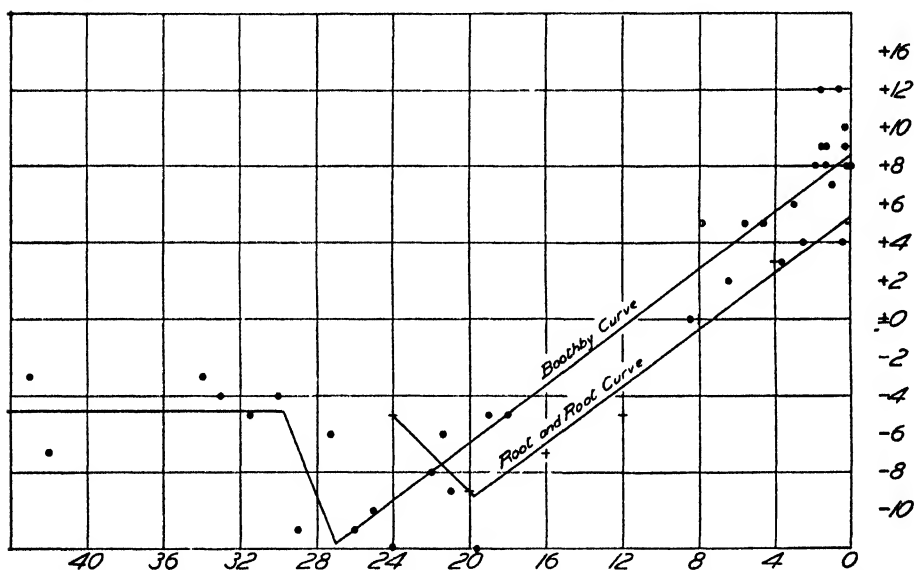
The results of these several manipulations are collected in the next table.

TABLE VI  
FOETAL HEAT PRODUCTION PER KILOGRAM COMBINED CURVE ONLY

Weeks	Calories			Foetal Weight	Calories per kilogram		
	I	II	III		I	II	III
25	—	—	—	0.07	—	—	—
23	14	15	16	0.13	108	115	123
21	30	31	32	0.22	136	141	145
19	46	47	49	0.35	131	134	140
17	62	64	67	0.51	122	125	129
15	77	81	84	0.70	110	116	120
13	93	98	102	0.93	100	105	110
11	109	114	120	1.19	92	96	101
9	124	131	139	1.50	83	87	93
7	140	148	158	1.83	77	81	86
5	156	167	178	2.20	71	76	81
3	172	185	198	2.61	66	71	76
1	187	202	216	3.05	61	66	71

Obviously, there is no suggestion of constancy in the weight-energy relation. A similar computation for the maternal organism correlating the maternal heat production calculated from weight increase with and without correction for foetal weight shows a variation between the extreme values of less than 10 per cent. Further, the individual maternal value per kilogram one week before delivery is only about one-third of that calculated for the foetus per kilogram at the same interval. Benedict and Benedict (13) have shown that the influence of a bath at neutral (body) temperature is to raise the oxygen consumption approximately 10 per cent in the majority of cases. This is of a very different order from the nearly 200 per cent difference between maternal (environmental) and foetal heat production per kilogram as recorded above.

But one other question comes to immediate attention. Prior to conception the normal healthy woman should and does have an energy requirement closely in accord with prediction. After she conceives, all of the existing observations show that at some point fairly early in the pregnancy there is a drop in the oxygen requirement to a level which may exceed the conventional lower normal limit of  $-10$  per cent. Further, subsequent to this drop there is a steady progressive linear rise reaching a level somewhat above prediction prior to delivery. In the next graph is



GRAPH 5. Progressive change in oxygen requirement during pregnancy showing period of readjustment of the gestating organism.

given a curve plotted from the data of Boothby and Sandiford (5) and also one from the observations reported by Root and Root (14), likewise on the study of a single case.

In the Boothby and Sandiford curve the inversion comes somewhere between the 30th and 26th week before delivery while the Root and Root depression seemingly occurs between the 24th and 20th week. In our own series fourteen of the patients were studied over periods of sufficient duration to permit a record of this period of transformation. In all of them it fell between the 30th and 22nd week before delivery, the estimated average mid point lying in the neighborhood of the 25th to 26th week. A full discussion of the implications of these observations is scarcely germane to the present text and will be considered elsewhere by one of us (15). While the

gestational period is highly variable and viable children may be delivered after pregnancies ranging from 33 to 47 weeks, the great majority terminate after an apparent span of 38 to 39 weeks. On the basis of the averages, the period of involution falls between the middle of the 3rd and that of the 4th lunar months. While one may concede the possibility of an active change in hormone control or other agency during the earlier weeks, it is hard to believe that the tiny protoplasmic mass of the foetus *per se* can exercise any very significant influence on the energy requirement of the total organism. At 27 weeks before delivery the estimated weight is 30 grams, the surface area 0.006 square meters. There is also the possible influence of the placenta to be considered. Benoist (16) has stated that up to 3.5 lunar months this tissue outweighs the foetus though at delivery it usually is no more than 20 per cent of the weight of the new-born child.

One last consideration may be touched on briefly. From the curves just discussed it is obvious that after conception has taken place, for a time at least, the relative oxygen consumption of the maternal organism is apparently unaffected. Then within the space of a few weeks a marked lowering from prediction occurs producing a level to be regarded as hypofunctional in character. Thereafter, a progressive recovery is manifest which continues to the end of gestation. This could be attributed to specific foetal influence, to a spontaneous gradual recovery of the maternal organism from some depressing hormonal or other influence, or to a wide variety of other possible agencies. Assuming, once more, that the foetal influence is paramount and that it first manifests itself significantly at some time within this involution period, the progressive heat production can be readily computed from a series of intermediate steps, each assumed as the point of initial foetal influence. To compute this, the total metabolism as actually observed is taken as the starting point. The maternal contribution is then calculated from the weight increase, using the Harris-Benedict prediction with foetal weight deducted and Aub-DuBois prediction from area calculated from total weight, in other words, uncorrected for the foetus. If, for example, the observed basal rate of the mother is 10 per cent below prediction twenty-nine weeks before delivery, it is assumed that she maintains this relation throughout the remainder of the gestation. The total foetal contribution can then be computed by deducting the maternal metabolism as calculated from the total metabolism as observed. This difference, which is the assumed foetal contribution, is then correlated with the foetal area and the value per square meter computed. Such a series of data is collected in the next table.

Again there is a real lack of such constancy as might be anticipated were

TABLE VII  
CALORIES FROM FOETUS ALONE CALCULATED ON BASIS OF HEAT PRODUCTION PER  
SQ. METER OF FOETAL AREA PER 24 HOURS  
MATERNAL CONTRIBUTION CALCULATED USING HARRIS-BENEDICT CORRECTED AND  
AUB-DUBOIS UNCORRECTED PREDICTION VALUES

Combined Groups					
Weeks	29	27	25	23	21
29	0	—	—	—	—
27	3000	0	—	—	—
25	2833	1333	0	—	—
23	2550	1600	750	0	—
21	2267	1600	1033	567	0
19	2025	1500	1119	762	381
17	1855	1455	1164	890	600
15	1714	1414	1157	971	729
13	1575	1334	1127	966	782
11	1457	1248	1086	943	790
9	1380	1192	1048	928	800
7	1280	1137	1013	918	801
5	1227	1107	994	916	814
3	1175	1073	979	899	809
1	1122	1038	955	882	802

the foetal metabolism the sole or even the primary factor in producing the demonstrated excess heat production.

### DISCUSSION

Adding the foetal area to that of the mother reduces the variation in the heat production per square meter in the last 6 months from 13 per cent to about 3 per cent (see Table IV). On this basis it has been concluded that the increasing foetal metabolism is the factor determining the apparent increasing over-production of energy when the calculation is based upon the maternal organism alone. But if the arithmetical processes involved be considered, it will be found that a small, variable, progressive, corrective factor has been added to a much larger quantity which is also showing increase but at a slower rate. Any other biometric entity showing a similar change could be manipulated to give equally concordant results. For example, one of us (17) has shown that the weight of the average individual can be calculated from the hip circumference with a very fair degree of precision. When the formula is applied to the pregnant woman, at least in the later months of gestation, it gives values from 5 per cent to 8 per cent too low as the pelvic contents represents a localized weight increment not

affecting the hip girth. By a suitable arithmetical manipulation of this minor variable (i.e., increasing hip girth) as a corrective factor a relatively uniform heat production throughout gestation could be apparently demonstrated. This is, of course, purely artifactual; the later analyses in this paper of the foetal influence would seem to indicate that the apparent constancy of the heat production in terms of the combined areas derives from a similar origin. Further, the normal heat production of the maternal organism with the age range of this series would be 37 to 38 calories per square meter per hour (3). The figures actually obtained are from 33 to 35 calories. The heat production of the new-born as measured by Benedict and Talbot (18) ranges from 27 to 36 calories. With the relatively small contribution made by the foetus to the combined surface, the foetal production would have to be of the order of 10 to 15 calories per square meter per hour one week before birth to account for the values recorded in Table IV if maternal consumption were normal. To conclude then, the deduction that the excess increment in the respiratory metabolism during the last 6 lunar months of pregnancy is due solely to the foetal metabolism would seem not to be warranted in fact. That the foetal metabolism plays its part no one could deny, but the special activity of this particular tissue mass is absorbed, in part at least, by the biometric magnitudes used as correlation factors for the entire organism. In like manner the placental activity is absorbed as well as those other tissues concerned with the storage of nitrogen which is a feature of this physiological condition (1, d). Further, there is the marked drop in the curve between 30 and 22 weeks which is unexplained by the limitation of active processes to the single one of foetal development. Seemingly, the mechanism is not so simple as was at first concluded and other forces certainly play a part, initially in lowering the basal rate to a subnormal level, and later conditioning a progressive return to a point superior to that of the initial downward departure.

#### SUMMARY

This paper may be briefly summarized as follows:

1. The heat production of a series of 77 women throughout the last 6 lunar months of normal pregnancy is analyzed.
2. It is demonstrated that during the 3rd to 4th month of gestation there is a rapid decline in the energy requirement from a normal to a subnormal level, the latter reached in about 4 weeks.
3. From this point on, during the last 6 lunar months there is a steady increase in the basal metabolic rate amounting to 13 per cent or more in excess of that conditioned by the gross increase in body weight.

4. The conclusion that this excess is contributed primarily by the foetus has been analyzed and seemingly found not to be supported by fact. The constancy of heat output in terms of surface area when the foetal area is added to that of the mother is apparently artifactual and results from the application of a small progressive factor as a correction to a larger quantity also showing progressive incremental change but at a slower rate.

5. The excess heat production is apparently the result of a complicated and unknown mechanism, engendered by the state of pregnancy but involving other factors than those of foetal tissue growth alone.

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## THE PROTEIN REQUIREMENTS OF THE ALBINO MOUSE

By

FRANKLIN C. BING, W. LLOYD ADAMS AND RUSSEL O. BOWMAN

*(From the Department of Biochemistry, School of Medicine,  
Western Reserve University, Cleveland.)*

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THE experiments of Wheeler (11) seemed to indicate that the mouse requires a higher level of dietary protein than does the rat. Using the so-called synthetic type of food mixtures, Beard (2) claimed the growth of the mouse was stunted with diets containing less than about 23 per cent of the total calories in the form of a complete protein.<sup>1</sup> It may be remarked, however, that even though most of the recent nutrition studies with mice have been made with diets containing about 25 per cent casein, the protein concentration of the usual rations fed to breeding colonies has been considerably less. Dawburn (3) has reported good growth with a diet containing 15.6 per cent casein. A careful reading of the published data has revealed that both Wheeler and Beard based their conclusions on the results obtained with very few animals, and Dawburn did not include experiments with higher or lower protein concentrations for comparison with the figures obtained with the 15.6 per cent casein diet. For certain studies on the nutrition of the mouse we found it desirable to feed a diet containing minimal amounts of casein and it was important to know how far the protein could be reduced in amount before it became the limiting factor for growth. There are, naturally enough, other reasons why it would be desirable to know, more precisely than at present, the protein requirements of the smallest mammal used in nutritional investigations. It seemed, for example, that a comparison of the requirements of the mouse—once these were determined—with the known requirements of the rat might throw some light on the problem of variations due to species, particularly those that might be attributed chiefly to differences in body size.

This paper presents data on the early growth of mice fed upon synthetic diets of varying casein content. Osborne and Mendel (9) showed that cystine became the limiting factor for the growth of rats restricted to diets containing low concentrations of this protein. Lightbody and Lewis (6) and Beadles, Braman and Mitchell (1) have demonstrated that such diets

<sup>1</sup> In this paper, unless otherwise stated, dietary protein is expressed in terms of percentages of the total calories yielded by protein.

also produce a poor growth of hair. We have made some determinations of the glutathione concentration of muscle and liver on the assumption, first, that the iodometric titration is really a measure of this substance and, second, that the production of tissue glutathione is in part limited by the supply of cystine in the diet. Accordingly the determination of tissue glutathione would indicate the effect of the diet upon what Lightbody and Lewis have termed the "more essential tissues" in contradistinction to a "less essential epidermal structure" such as hair.

### EXPERIMENTS

Mice of the inbred Bagg albino strain, reared in the laboratory, were removed from their mothers when three weeks old and placed in separate, false-bottom cages. They were fed the diets, described in Table I, for four

TABLE I  
COMPOSITION OF THE DIETS

Diet No.	0.95	1.96	4.0	7.8	15.6	23.2	49.7
Component	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Casein	12.5	25	50	100	200	300	670
Salt Mix.	40.0	40	40	40	40	40	40
Cornstarch	359.0	353	340	315	265	215	—
Sucrose	298.5	292	280	255	205	155	—
Lard	200.0	200	200	200	200	200	200

McCollum's salt mixture (*Jour. Biol. Chem.*, 1918, 33, 55) was used, to each 100 grams of which were added 2.81 gm. of  $\text{Al}_2(\text{SO}_4)_3 \cdot \text{K}_2\text{SO}_4 \cdot 24\text{H}_2\text{O}$ , 0.35 gm. of NaF, 0.66 gm. of  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$  and 0.21 gm. of KI.

150 mg. of dried yeast and 120 mg. of cod liver oil were also administered to each mouse daily apart from the rest of the diet.

weeks, measurements of food intakes and body weights being recorded at weekly intervals. An observation period of four weeks' duration was chosen for the following reasons. It comprises the time during which the most rapid growth following weaning takes place; differences in growth rate due to sex have not become evident, and the data for males and females may be combined; and, finally, there is not the excessive fat formation that occurs sometimes in older animals and interferes with the interpretation of increases in body weight. It is also probable that limiting the period of observation would accentuate the high requirements postulated by Wheeler and Beard. Thus, Smith and Möise (10) have found that young rats may show at first a better growth if the dietary protein level is increased to 38 per cent but, if the experiment is prolonged, rats fed upon 12 per cent protein thrive somewhat better.

The protein used in the preparation of the diets was a good grade of commercial casein that was subsequently percolated several times with 0.2 per cent acetic acid to remove salts and other soluble impurities. The resulting air-dried product contained 13.2 per cent nitrogen.

Both yeast and cod liver oil were administered separately from the rest of the diet. The dried yeast was fed at a level of 150 mg. per mouse per day, a preliminary assay having shown this amount to be more than sufficient for normal growth. The preparation contained 7.05 per cent nitrogen, an amount sufficiently high to introduce a possible source of error in the experiments. The following attempt was made to evaluate this nitrogen. A group of 15 young mice were stunted by restriction to a diet low in casein. When the weight remained practically constant the dried yeast was increased to 300 mg. per day, but no change in weight during a period of one week was noted. The yeast was thereupon restored to its former level and supplements of casein were administered to some animals, cystine to others, while three mice were reserved for controls. The latter group barely maintained weight, but appreciable growth occurred in those mice receiving daily supplements for one week of either 50 mg. of casein or 1.0 mg. of cystine. It was concluded that the effect of the nitrogenous compounds of the yeast, in the amount fed, could for the present purposes be neglected.

While the weight of basal food eaten varied somewhat for individual mice, the intakes of yeast and cod liver oil were constant. Calculating the percentage of protein by weight in terms of total food intake, including the vitamin-containing adjuvants, the diets furnished 1.20, 2.47, 4.9, 9.8, 19.7, 29.2 and 63.0 per cent casein, respectively. Expressed as percentages of the total calories eaten, these average protein values become 0.95, 1.96, 4.0, 7.8, 15.6, 23.2 and 49.7 per cent. In calculating the caloric values for casein, cornstarch, sucrose and dried yeast, the factor of 4 calories per gram has been used, and for lard and cod liver oil, 9 calories per gram. The food mixture referred to as "stock" consisted of whole ground wheat 65.8 per cent, dried whole milk 32.9 per cent, sodium chloride 0.65 per cent, and calcium carbonate 0.65 per cent.

The question may be asked whether the amount of salt mixture in the diets was sufficient, because along with the supposedly higher protein requirements it has been believed that the mouse needed a higher concentration of inorganic salts. We have found, however, in some unpublished experiments, that 4 grams of a complete salt mixture per 100 grams of dried food is more than adequate for normal body growth and normal bone production. Hence, we have used this concentration because we thought it

preferable and also because it conformed with the customary usage in nutrition studies with rats.

At the age of seven weeks the mice were killed by bleeding during ether anesthesia. The muscles of the hind legs were then dissected, weighed, and the sulfhydryl content determined by Hess' procedure (5) with one or two minor alterations, that follow. The protein in samples of tissue weighing 1 to 2 grams (representing, sometimes, several animals) was precipitated with tungstic acid, the titration with  $M/1200\text{ KIO}_3$  was done with a micro-burette, and two drops of freshly prepared starch solution were added as an indicator just before reaching the end-point. In a number of cases analyses were also made of the liver.

### RESULTS AND CONCLUSIONS

The data for males and females have been pooled because no differences attributable to sex could be detected. Animals that consumed insufficient food (less than 10 grams of the basal diet per week), due to failure to eat the yeast or for unknown reasons, have been omitted from the tabulations. These mice were chiefly from groups fed diets containing 0.95, 1.96 and 49.7 per cent protein. The data for most of the individual animals may be obtained from Figure 1, whereas the values in the tables are grouped averages. As a means of indicating the variation within each group, the standard deviations of the mean values for growth and food intake have also been determined.

*Food consumption.* Table II shows that the average weights of basal food eaten by the mice fed diets containing 1.96, 4.0, 7.8 and 15.6 per cent protein were remarkably constant, varying only from 63.2 to 64.8 grams

TABLE II  
GROWTH AND FOOD CONSUMPTION OF THE MICE

Diet Casein per cent	No. of mice	Av. Wt. beginning	Av. Wt. end	Av. Gain in Wt. in 4 weeks $\pm$ S. D.	Basal Food Intake in 4 weeks $\pm$ S. D.
		gm.	gm.	gm.	gm.
0.95	4	9.3	7.8	$-1.5 \pm 0.5$	$52.9 \pm 6.0$
1.96	6	9.4	10.1	$0.7 \pm 0.9$	$63.5 \pm 12.1$
4.0	12	8.6	11.1	$2.5 \pm 1.0$	$63.2 \pm 10.9$
7.8	10	9.1	15.4	$6.3 \pm 1.6$	$63.5 \pm 10.6$
15.6	14	9.3	17.0	$7.7 \pm 1.3$	$64.8 \pm 8.9$
23.2	8	8.1	15.7	$7.6 \pm 1.9$	$58.4 \pm 6.4$
49.7	3	7.7	15.6	$7.9 \pm 1.3$	$44.8 \pm 3.1$
Stock	9	7.2	15.9	$8.6 \pm 1.9$	— —

in four weeks. There seemed to be a tendency for animals receiving a very low protein diet (0.95 per cent), and those receiving a very high protein diet (49.7 per cent), to eat less food. The individual variations in both groups are large enough, however, to render the observed differences of little significance. The statistical method that we have used to calculate the significance of the mean results is that described by Fisher (4), and is

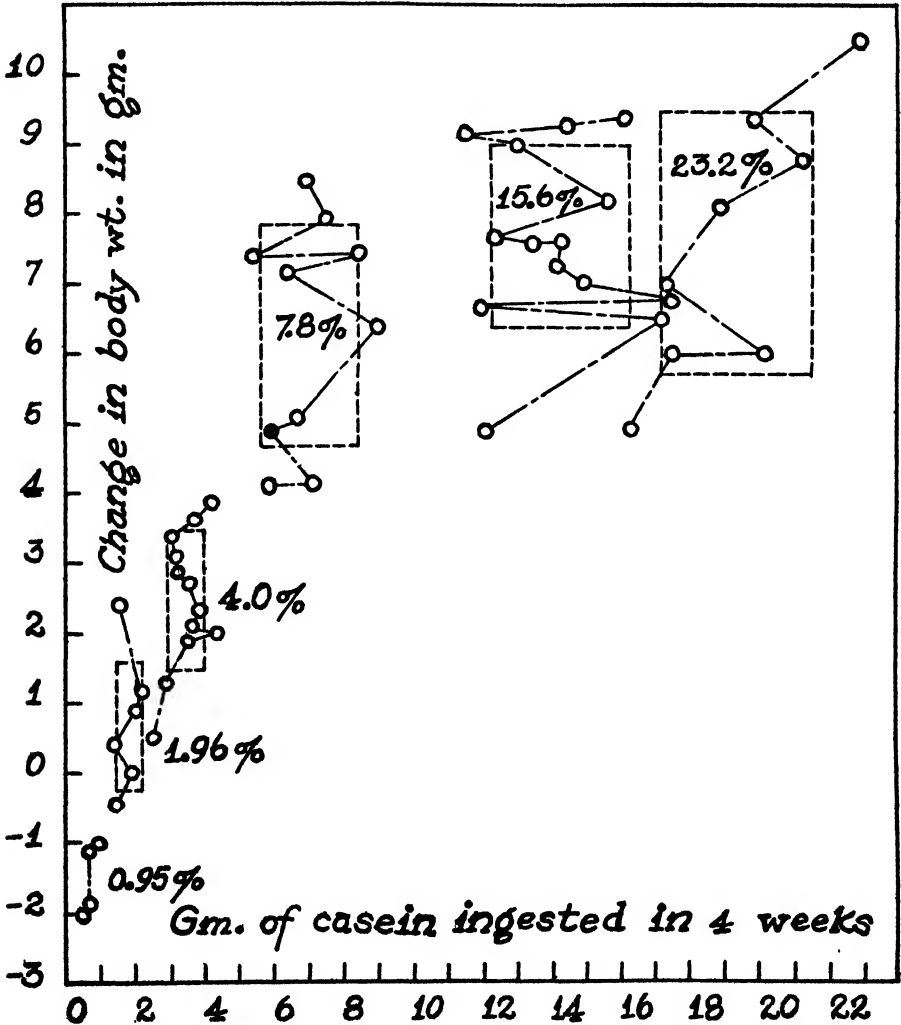


FIG. 1. The chart shows the relationship between dietary protein concentration, food intake (expressed as grams of casein eaten in 4 weeks), and the gain in weight. Each circle represents one mouse, and those animals receiving the same diet are shown connected by a dot and dash line. The dotted rectangles enclose the areas bounded by twice the standard deviation of the average growth of each group by twice the standard deviation of the average casein intake.

particularly adapted for treating small samples. To estimate Fisher's "t" the given values for the standard deviations may be converted into  $\Sigma d^2$  by squaring and multiplying by one less than the number of cases. This value can then be substituted in the equation developed by Fisher.

*Growth.* It may be noted that the level of protein in the diet containing 0.95 per cent casein is about equal to the fecal metabolic nitrogen (0.2 gm. per 100 gm. of dried food) shown by Mitchell (8) to be excreted by man, several species of farm mammals, the dog and the rat, when fed upon low residue diets. It is not surprising, therefore, that the mice receiving the lowest protein diet lost weight. Apparently, about 1.95 per cent protein is necessary for maintenance of weight, at least between the ages of three and seven weeks. Mice fed upon 4.0 per cent casein were stunted; the diet containing 7.8 per cent casein produced nearly normal growth; and the average gains in weight of mice fed diets containing 15.6, 23.2 and 49.7 per cent casein were practically identical. By Fisher's method it was calculated that the difference in average growth of mice fed 7.8 and 15.6 per cent casein, respectively, is real, though further experimental study of this point would be desirable.

The average gain of animals fed upon the stock diet was somewhat greater than the best average growth on synthetic diets, but it is difficult to say from the data available whether the slightly better growth is real or due to chance.

It may be concluded that the protein (casein) requirements for the normal growth of the mouse are certainly not more than 15.6, and perhaps somewhat more than 7.8, per cent. Regarding the efficiency of protein utilization it can be calculated that the mice fed 7.8 per cent casein showed the greatest gain per gram of ingested protein. Finally, it can also be calculated that the higher casein levels produced somewhat better growth in terms of gain per gram of basal food.

*Composition of the body as indicated by glutathione determinations on muscle and liver tissues.* The values for the sulfhydryl compounds present in muscle and liver are recorded in Table III as average percentages of reduced glutathione in the fresh tissues. These were somewhat higher than values we have found in rat muscle. The average concentration of glutathione in the muscles of mice receiving diets containing from 4.0 to 49.7 per cent casein, and those receiving stock rations, ranged from 0.052 to 0.064 per cent. In other words, it seemed that where growth occurred, even though it was stunted growth, normal glutathione concentrations were found in the muscles. One is tempted to conclude that if cystine be the limiting factor in the diet, muscle tissue of normal glutathione content is

produced or growth does not occur. At least, this conclusion would be in harmony with Liebig's law of the minimum. While our data on mice fed 0.95 and 1.96 per cent casein are too meager to permit any positive statements to be made, we believe further investigation would show that the dietary protein must be diminished to a bare maintenance level or less before a deficiency in muscle glutathione would occur.

TABLE III  
CONCENTRATION OF REDUCED GLUTATHIONE IN MUSCLE AND LIVER

Experimental Animals			
Diet No.	Av. Weight gm.	Av. Glutathione	
		Muscle per cent	Liver per cent
0.95	7.8	.024 (1)	.130 (1)
1.95	10.1	.040 (2)	.120 (2)
4.0	11.1	.064 (2)	— (0)
7.8	15.4	.059 (2)	.154 (1)
15.6	17.0	.056 (5)	.365 (1)
23.2	15.7	.059 (4)	.326 (2)
49.7	15.6	.061 (2)	.285 (1)
Normal Controls on Stock Diet			
Age			
3 weeks	8.0	.067 (3)	.310 (5)
7 weeks	15.9	.052 (5)	.302 (5)
Adults	22.0	.043 (4)	.310 (4)

The figures in parentheses refer to the number of determinations.

The concentration of sulfhydryl compounds in the liver seems to bear a less constant relation to growth. Mice fed upon 7.8 per cent casein, although they showed good growth, had about half as much liver glutathione (one determination only) as did normal animals. Marenzi and Lac-lau (7) have found a decrease in glutathione in the livers of rats fed upon low protein diets. Our results may indicate that the mice fed upon 7.8 per cent casein were growing under a condition of nutritional stress.

#### DISCUSSION

Concerning the level of dietary protein, the present results show that the requirements of the growing mouse are very similar to those of the growing rat. Both seem to require not less than about 15 per cent of their total



calories in the form of a complete protein in order to grow at the rate which is normal for the species. However, the possibility remains that further improvements in technic might show that somewhat less than 15 per cent protein calories may be satisfactory.

The average human dietary probably contains between 10 and 20 per cent protein. Computations of the protein content of the milk of 13 species show variations from 7 to 35 per cent, averaging 20 per cent, which is also the value for cow's milk. The fact that human milk contains only 7 to 9 per cent of its total calories in the form of protein has been interpreted as indicative of the low requirements said to be characteristic of slowly growing species, plus the exemption from the demands of growing hair, and the possibly higher nutritional value of the proteins, particularly the lactalbumin.

There seems to be little justification, in fact or in theory, for the supposition that the mouse should require a higher dietary level of protein because it attains mature body weight a little more quickly than the rat. If there are any higher requirements they seem to be fulfilled by the increased food consumption per gram of body weight that in turn is due to the higher rate of metabolism. Differences, then, become apparent when the protein needs are expressed, not in terms of dietary protein level, but in grams of protein necessary to produce a unit gain in weight, or the amount of protein required per kilogram of body weight to produce either maintenance or normal growth. On such bases as these, the smaller species will require proportionately more protein. Thus, 1 gram of casein will produce 0.5 to 0.9 gram of mouse, or about 2 grams of rat, and 1 gram of protein in the form of breast milk will produce about 5 grams of baby.

#### SUMMARY

Young mice were fed complete diets of the "synthetic" type in which the protein (casein) levels were, respectively, 0.95, 1.96, 4.0, 7.8, 15.6, 23.2 and 49.7 per cent of the total calories ingested. The food consumption and changes in body weight were measured between the ages of three and seven weeks. All the animals ate approximately equal quantities of food, except those fed the highest and lowest protein levels, respectively, where the intakes were less, though perhaps not significantly so. The mice fed 0.95 per cent casein lost weight, those fed 1.96 per cent maintained weight, and those fed 4.0 per cent grew at a definitely sub-normal rate. The mice fed 15.6, 23.2 and 49.7 per cent casein grew at nearly identical rates and attained almost the weight of animals fed upon a stock diet. Mice fed upon 7.8 per cent casein grew at a slightly sub-normal rate. The reduced gluta-

thione concentration in the muscles of the mice was about the same in all animals that grew, even though the growth might be stunted. The mice fed 7.8 per cent casein had about half as much glutathione in the liver as mice fed higher amounts of casein, indicating that their growth, which was also the most efficient in terms of gain in weight per gram of ingested casein, was under a condition of nutritional stress. It is concluded that, contrary to what has been believed, the protein requirements of the mouse are certainly fulfilled by diets containing 15.6 per cent casein, and probably somewhat less would also be satisfactory. These are about the same as the requirements of the rat, the differences due to metabolic rates becoming apparent when the gain in weight per gram of ingested protein is measured.

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## THE HEAT PRODUCTION OF UNUSUALLY LARGE RATS DURING PROLONGED FASTING

By

FRANCIS G. BENEDICT, KATHRYN HORST, AND LAFAYETTE B. MENDEL  
(*From the Nutrition Laboratory of the Carnegie Institution of Washington, Boston, and the Laboratory of the Connecticut Agricultural Experiment Station and the Laboratory of Physiological Chemistry, Yale University, New Haven.*)

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THE ability of small animals to withstand prolonged fasting is greater than has been commonly supposed. Thus, wild rats have survived without food for from 5 to 8 days (1) and laboratory-bred rats for 16 days or longer (2). In studies with rats at Yale University on the significance of various diets and diet ingredients, some unique rats were developed. The configuration of these rats did not suggest any abnormality in body composition, such as an unusually large deposition of fat. Consequently one would not expect the very large rat to have an advantage over the normal-sized rat in the ability to withstand fasting because of a greater initial reserve of bodily energy. If there were enormous deposits of fat, such as are found in animals that are about to hibernate, or in geese and pigs that have been purposely stuffed, one might expect longer resistance to fasting. An investigation was therefore planned to study the physiology of two very large rats (maximum weights 830 and 766 grams) during prolonged fasting at thermic neutrality. At frequent intervals throughout the fasts observations were made of the loss in body weight, the rectal temperature, and, as an index of the level of vital activity, of the oxygen consumption. As the metabolism of the very large rats was measured under basal conditions, i.e., on the first day of the fast, data were at hand for a comparison with the basal heat production of smaller rats. Hence, in addition, basal metabolism measurements at thermic neutrality were made on a number of other full-grown rats, some medium in size (307 to 396 grams) and others large (407 to 562 grams).

### THE ANIMALS, THEIR DIETARY HISTORY AND RATES OF GROWTH

Male albino rats from the Osborne and Mendel colony were employed. With the exception of rat No. 25, all the animals had been used for a study of the influence of the rate of growth upon metabolism. As the ultimate weights attained by the rats varied with the kinds of diet and the rates of growth, these rats, when full-grown, were employed for control purposes in

the study of the *basal* metabolism of the unusually large rats. The medium-sized rats, Nos. 1 to 12, had gained weight at the rate of about 2 grams a day during the normal period of growth. These rats were fed a synthetic diet,<sup>1</sup> with daily supplements of yeast in amounts to secure the desired increment in weight. At the time that the metabolism measurements concerned with size were made, the medium-sized rats had subsisted upon the experimental rations, on the average, about 230 days. The large rats, Nos. 13 to 24, had gained about 4 grams a day during the period of most rapid growth. These rats were fed a synthetic diet<sup>2</sup> supplemented daily with dry brewers' yeast (200 to 400 mg.) and 20 grams of fresh lettuce. The large rats had received the special diets for about 250 days when the metabolism measurements under discussion were made. Some of the "slow growth" rats and some of the "rapid growth" rats received cod liver oil, but this adjunct was not universally fed.

The two unusually large rats each had a special history. Rat No. 26 gained on the average 5.0 grams per day for 55 days, between the age of 23 and 78 days. Although the average daily gain of this rat did not exceed the best growth records that have been reported for the normal rat (6.1 grams (3) and 7.3 grams (4) during the period of growth from 60 to 300 grams), a gain of 276 grams in 55 days is an exceptional weight increment. At first, the diet of rat No. 26 (23 to 331 days) was similar to that of rats Nos. 13 to 24. With this diet, growth ceased when the animal weighed 664 grams. Then, for the next 96 days, the rat received rations consisting of a "special" dog biscuit, wheat germ food<sup>3</sup> and fresh lettuce. Occasionally the wheat germ food was replaced by milk food.<sup>4</sup> During the first 63 days of this dietary regimen, the weight of the rat increased from 664 to 820 grams. During the last 33 days the weight fluctuated between 810 and 830 grams. The other very large rat, No. 25, had been fed dog biscuit and a variety of other foods, including lettuce and bananas. The growth rate of this rat was not known, nor was there information concerning the exact age of the rat. The animal was probably in the second year when the metabolism was studied.

All the rats except No. 25 were brought to the metabolism laboratory when about 30 days of age. They were housed separately in cylindrical metal cages (5). The temperature of the environment ranged between 23°

<sup>1</sup> Casein, 18 per cent; Osborne and Mendel salt mixture, 4 per cent; cornstarch, 54 per cent; butter fat, 9 per cent; lard, 15 per cent.

<sup>2</sup> Casein, 35 per cent; Osborne and Mendel salt mixture, 4 per cent; cornstarch, 37 per cent; butter fat, 9 per cent; lard, 15 per cent.

<sup>3</sup> Whole milk powder, 50 per cent; wheat germ, 20 per cent; lard, 30 per cent.

<sup>4</sup> Whole milk powder, 60 per cent; cornstarch, 12 per cent; lard, 28 per cent.

and 25°C. Rat No. 25 had not been confined in a small cage during the major portion of its life, but had wandered about in a large room during the day. Overnight it was confined in a standard rat cage. Rat No. 25 was brought to the metabolism laboratory 20 days before the long fast and 8 days before the first metabolism measurement. During this 20-day period, rat No. 25 was caged like the other rats. It lost 36 grams in weight during the period of acclimatization to the metabolism laboratory.

Rat No. 26 began its prolonged fast when it was 430 days old. It fasted from December 18 to February 13. During the fasting period it was kept in a special room at a constant temperature (26° to 28°C.). Water was available at all times, except during metabolism measurements. A raised screen of coarse wire mesh was placed in the rat cage for the first 5 days of the fast, i.e., the period during which food residues might be excreted. Thereafter the screen was not used. It was our intention to let rat No. 26 fast until death, but the importance of making certain histological studies seemed to warrant killing the animal on the 57th day of the fast, before it actually succumbed. The autopsy was performed immediately, and the findings are noted subsequently in this report. Rat No. 25 fasted from June 9 to July 17 at the prevailing environmental temperature. During the long fast it was caged like rat No. 26 and also had access to water. With rat No. 25 the plan was to refeed the animal and then initiate a second fast. The purpose of realimentation was to determine whether the rat would regain its initial weight and at what rate, and whether it could then withstand a second fast as long as the first. Hence, on the 38th day of the fast, when the animal had lost 45 per cent of its initial weight (730 grams), food<sup>5</sup> was again given. At this time the rat appeared well and in a normal condition, without external evidences of debilitation or disease. After the 44th day of realimentation the rat, without apparent cause, suddenly began to lose weight and died 9 days later. A post mortem examination did not reveal any anatomical abnormalities.

#### TECHNIC

The oxygen consumption was measured with the multiple-chamber respiration apparatus previously described (6). Owing to the great size of the animal, a special chamber was built for rat No. 26. The rat was left inside the respiration chamber for adjustment for approximately one hour. The exact time required for the animal to absorb from the closed ventilating circuit a known volume of oxygen (about 370 cc.) was determined for 3

<sup>5</sup> The diet consisted of dog biscuit, milk food or wheat germ food, and lettuce.

or more successive periods, these periods varying in length from about 40 to 90 minutes. In the final tabular presentation of results the average of 3, occasionally 2, and rarely 4 successive determinations has been used. The data are reported in the following tables as the oxygen consumption per 6 hours, the heat production per kilogram of body weight, and the heat production per square meter of body surface per 24 hours. Since it is known that the respiratory quotient of the fasting rat is essentially 0.72, the heat production has been calculated from the measured oxygen consumption on the assumption that for each liter of oxygen consumed 4.702 calories are produced. In the calculation of the surface area the Rubner formula  $S = 9.1 \times w^{2/3}$  was used.

Theoretically, strict comparisons of metabolism measurements are possible only when animals are completely immobile. That with rats is impossible, although we have found that there is a period between 10 A.M. and 4 P.M. when rats are usually quiet. The metabolism measurements, both in the basal periods 24 hours after food and during the prolonged fasts, were always made between these hours. The usual technic of recording graphically the degree of repose or activity of the rat was employed. With the large rats, the activity was frequently not recorded so that a large animal cage could be used in the regular respiration chamber. For this reason, activity records were not secured during the basal metabolism measurements of some of the large rats (407 to 562 grams), nor was the activity of rat No. 25 recorded during metabolism measurements early in the long fast. From the kymograph records we have estimated, in accordance with our usual procedure, the percentage of time during the experimental period when the animal was active. These estimates of activity are recorded in the tables in all instances where they were obtained. Although it is impracticable to compare the activity in the different experiments too closely from the quantitative standpoint, it was obvious that the rats as a whole were not extremely active inside the respiration chamber. It is difficult to evaluate to what extent such chamber activity influences the metabolism. Although our estimates of activity are given a quasi-mathematical relationship by being expressed as percentages, these estimates must be taken with considerable reserve.

For metabolism measurements under basal conditions, food was withdrawn 24 hours prior to the experiment. During this interval the fasting rats lived in a special glass cage at a temperature not far from 28°C. The metabolism was measured at 30°C., both in the basal periods 24 hours after food and during the prolonged fasts. Each rat was weighed at the close of its metabolism measurement. The rectal temperature was determined

usually within 15 minutes after the end of the experiment. Observations upon the rectal temperatures were made with a one-minute clinical thermometer. With most small animals the determination of the body temperature is difficult. There is usually a great deal of struggle on the part of the animal, and consequently a momentary increase in temperature caused by the activity. There is likewise a cooling effect produced in turning the animal over on its back and exposing its abdomen to the air. Our animals were gentle, however, and especially advantageous conditions were obtained for securing the actual body temperatures. Thus, the rats were handled by a laboratory caretaker, accustomed to holding them. Feces, when present, were first expelled from the rectum by gentle pressure. The thermometer, well lubricated, was then inserted to a depth of at least 4 cm., and read at the end of one minute. The animals seldom struggled during the entire procedure.

#### DURATION OF FASTS; LOSSES IN BODY WEIGHT

The body-weight changes of our two very large rats during the periods of prolonged fasting are shown in Table I. Rat No. 26, which weighed 822 grams at the beginning of the fast, lived until the 57th day when it was killed and autopsied. On this day the weight had reached 376 grams, which was only 46 per cent of its initial weight. The average daily losses in weight and the per cent daily losses have been computed for both rats. The immediately preceding weight has been used each time as the baseline. On the first day of fasting rat No. 26 lost 25 grams, or 3.0 per cent of its weight. Rat No. 25 lost almost the same amount, 24 grams, but since it weighed 730 grams at the start, this corresponded to a loss of 3.3 per cent per day. The daily rate of loss remained relatively constant with rat No. 26, always a little over 1 per cent, until the 46th day of the fast. From then on, with the exception of the 50th day when the animal may have eaten feces from another rat, the percentage decrease in weight was somewhat greater.

Rat No. 25 weighed 730 grams when food was withdrawn and 398 grams on the 38th day of the fast, or 55 per cent of its initial weight. With this rat, after the first few days, the loss remained relatively constant from day to day at about 7 or 8 grams, approximating about 1.6 per cent daily of the preceding weight. When rat No. 26 had been without food for 40 days, its weight was 60 per cent of its initial weight. In other words, rat No. 26 was losing weight less rapidly than rat No. 25. Several factors may have contributed to this conservation of body tissue. In the first place, rat No. 26, although larger than rat No. 25, had a lower total metabolism (see



Table III). Secondly, rat No. 26 was inactive throughout the entire fast, whereas rat No. 25 was more restless. Cage life was relatively new for rat No. 25. This factor possibly contributed to the greater degree of restlessness of this rat. The environmental temperature was also controlled to within narrow limits for rat No. 26, whereas rat No. 25 was exposed to the prevailing environmental temperatures (summer) save when in the respiration chamber.

TABLE I  
BODY WEIGHT CHANGES OF TWO VERY LARGE MALE RATS DURING PROLONGED FASTING

Rat No. 26				Rat No. 25			
Duration of fast	Weight of rat	Average daily loss	Per cent daily loss	Duration of fast	Weight of rat	Average daily loss	Per cent daily loss
days	gm.	gm.		days	gm.	gm.	
0	822	—	—	0	730	—	—
1	797	25	3.0	1	706	24	3.3
5	748	12	1.5	3	685	11	1.6
9	716	8	1.1	8	637	10	1.5
12	688	9	1.3	10	623	7	1.1
15	665	8	1.2	15	580	9	1.4
19	634	8	1.2	17	562	9	1.6
22	612	7	1.1	22	524	8	1.4
26	588	6	1.0	24	512	6	1.1
29	566	7	1.2	29	473	8	1.6
33	544	6	1.1	32	440	11	2.3
36	516	9	1.7	35	422	6	1.4
40	494	6	1.2	38	398	8	1.9
43	476	6	1.2				
46	452	8	1.7				
48	433	10	2.2				
50	*427	3	0.7				
52	410	9	2.1				
54	397	7	1.7				
56	382	8	2.0				
57	376	6	1.6				

\* Two days before this weight was determined rat No. 26 was photographed. Possibly the animal ate feces from another rat during the time it was not in its own cage.

The draft upon body weight was more rapid and heavier toward the end of the fast with both animals, particularly with rat No. 26. Since fasting organisms in general tend to become richer in water, this is all the more significant. Indeed, one might expect that in these two cases the protein storage was being drawn upon, although the autopsy of rat No. 26 showed a liberal reserve of fat. This finding makes it particularly unfortunate that the nitrogen excretion was not directly determined.

Since the total life span of the laboratory-bred albino rat rarely exceeds three years, our rat that fasted 57 days was without food for about one-twentieth of its probable total life. This fasting period with the rat would correspond to 4 1/2 years without food in the average life span of man.

*Resistance to fasting (autopsy findings).* As the fasts progressed, it was obvious that these very large rats were capable of withstanding complete withdrawal of food far longer than anyone would have predicted. There were no evidences of debility and the animals were in no sense approaching a moribund state. The generally good condition of rat No. 26, even at the termination of the fast, was confirmed by a post mortem examination,<sup>6</sup> which showed that after 57 days without food considerable quantities of mesenteric and perirenal fat were still present. All the fat of the omentum was gone and the membrane had degenerated. The cause of the peculiar eye condition of rat No. 26 was diagnosed by a special study of the tissues. There was no evidence of vitamin A deficiency. The eyes were sunken into their orbits and showed a serosanguineous discharge. The Harderian glands were atrophied. With the exception of these degenerative changes, the rat was still in good condition. The lungs were normal, the heart was not enlarged, the liver was pale in color and large, but not any greater in size than has usually been observed in "rapid growth" rats. The kidneys were congested and large; the testes small and flaccid. The stomach showed hypertrophy; the glands at the base of the tongue were normal. A histological examination of the tissues of the various organs was made.<sup>7</sup> The tissues were compared with those of rats that had a similar dietary history but had not been subjected to a long fast. The thyroid was normal. A moderate amount of colloid was present with the usual variation. There were not any greatly enlarged acini nor areas of hyperplasia without colloid. The liver was greatly altered. The cytoplasm formed a net resembling a honeycomb with nuclei scattered through this apparent syncytium. The testes were greatly atrophied with no sign of spermatogenesis. The diameter of the seminiferous tubules was small. In the adrenal there was perhaps a slight tendency toward cytoplasmic vacuolization in the medulla. The kidney showed vacuolization of the cells of the convoluted tubules and also in the collecting ducts.

*Realimentation after prolonged fasting.* During the first 44 days of realimentation, rat No. 25 increased in weight from 398 to 592 grams, repre-

<sup>6</sup> Dr. A. M. Yudkin, of the School of Medicine of Yale University, kindly performed the autopsy.

<sup>7</sup> The authors are indebted to G. B. Moment of the Osborn Zoological Laboratory, Yale University, for the histological studies.

senting an average daily increment of 4.4 grams. This rate of gain during the recovery from the prolonged fast compares favorably with the optimum rate of growth in *normal young male rats*, with which daily increments of from 6.1 to 4.8 grams have been found during the period of growth from 60 to 300 grams (7). Moreover it approaches that found in *resumed* growth after long periods of suppression by inadequate diets. Mendel and Cannon (7), for example, in a recalculation of earlier results of Osborne and Mendel (8), have reported daily increments of from 7.6 to 5.9 grams in such instances.

#### THE BASAL METABOLISM OF FULL-GROWN MALE RATS OF VARIOUS SIZES

Our basal metabolism measurements on full-grown male rats, not only those of normal size but particularly those of unusually large size, are recorded in Table II. We have made no attempt to collect all the gaseous metabolism data on male rats published in the literature heretofore, but present our own measurements here primarily so that we may have, for comparison with the very large rats, observations on normal-sized rats from the same colony, living under precisely the same conditions. The basal metabolism of all the rats except No. 25 was measured several times during the period of growth and again when maturity was reached. Only the measurements obtained on mature rats are recorded in Table II. In many instances two measurements, 20 days apart, were made while the rats remained stationary in weight. Since the two determinations usually agreed well, only one result is presented in Table II. The metabolism of rat No. 25 was determined twice under basal conditions, once 12 days before the fast and again on the first day of the long fast. The basal metabolism of rat No. 26 was measured only once after its maximum size (830 grams) had been attained. This determination was made on the first day of the long fast.

The effect of size *per se* upon the metabolism is strikingly shown by the data in Table II. With animals of the same species it is commonly found that the smaller animal has the larger heat production per unit of weight. This is the case with the rats listed in Table II. Thus, the medium-sized rats weighing from 307 to 396 grams produced on the average 91.8 calories (range from 80.5 to 100.0 calories) per kilogram of body weight per 24 hours, whereas the larger rats weighing from 407 to 562 grams produced on the average 77.4 calories (range from 69.0 to 83.0 calories). It is usually assumed that the calculation of the heat production per unit of surface area eliminates the effect of differences in size, but even on this basis the larger rats have a definitely lower heat production than the normal-sized

rats. The larger rats showed a heat production varying from 587 to 731 calories per square meter of body surface, or on the average 664 calories. The values for the medium-sized rats varied from 615 to 774 calories, and averaged 708 calories.

The basal heat production of the two unusually large rats, Nos. 25 and

TABLE II  
BASAL METABOLISM OF FULL-GROWN MALE RATS OF VARIOUS SIZES  
(Measured at 30° C. and 24 hours after food.)

Rat No.	Age	Body* weight	Rectal temp.	Heat production per 24 hours		Activity
				Per kg.	Per sq. m.	
	days	gm.	°C.	cal.	cal.	%
1	232	307	37.2	100.0	740	12
2	285	308	37.9	92.5	687	—
3	204	313	37.9	97.5	726	22
4	170	323	38.4	94.5	714	11
5	239	338	37.9	80.5	615	10
6	267	338	37.6	88.5	676	18
7	302	344	38.2	91.0	700	—
8	302	364	37.8	86.0	676	3
9	273	372	36.9	88.5	699	10
10	272	378	37.8	90.5	717	11
11	277	378	37.0	96.5	766	—
12	281	396	36.8	96.0	774	1
Average	—	—	37.6	91.8	708	—
13	330	407	38.5	81.5	664	—
14	224	429	37.4	77.5	643	8
15	274	434	37.6	75.5	626	7
16	294	440	37.2	74.0	620	7
17	249	456	36.8	82.5	699	—
18	325	463	35.3	69.0	587	—
19	251	465	37.7	78.0	667	9
20	299	473	37.9	77.5	661	6
21	281	518	37.9	83.0	731	—
22	266	538	37.2	81.0	722	—
23	288	557	38.3	76.5	690	—
24	269	562	37.8	73.0	661	—
Average	—	—	37.5	77.4	664	—
25	—	723	38.0	63.0	621	—
	—	706	37.4	73.5	717	—
26	430	797	37.4	59.0	603	—

\* The length from nose to anus was, on the average, 24.7 cm. for rats 1 to 12, and 26.4 cm. for rats 13 to 24. The length of rat No. 26 was 28.7 cm. The length of rat No. 25 was not determined.

26, was lower than that of the other two groups of rats. Thus, per kilogram of body weight the heat production of rat No. 26 was 59 calories or lower even than the minimum value of 69 calories for rat No. 18 in the group of large-sized rats. Similarly, rat No. 25 on this same basis had a heat production of 63 calories, which is measurably lower than the minimum value for rat No. 18. On the other hand, when the basal metabolism of rat No. 25 was measured a second time, a higher value of 73.5 calories was noted. This is substantiated by another high value on the 8th day of fasting (see Table III). The highest basal value for rat No. 25, however, is lower than the average of 77.4 calories for the group of large-sized rats in Table II, and the picture is consistent in that the larger the rat the lower is the heat production per unit of body weight. On the body-surface basis likewise the unusually large rats, in general, have a lower heat production than either the medium-sized or large-sized rats, 603 and 621 calories as compared with 708 and 664 calories. Rat No. 18, weighing 463 grams, is an exception. This rat actually had as low a heat production as 587 calories. But the data in general in Table II indicate a trend for a lower metabolism per unit of surface area, the larger the rat.<sup>8</sup>

Although there is a certain degree of lability in the rectal temperatures of normal rats and many observers have had difficulty in obtaining them, there is nothing in the picture of the temperature of these very large rats to indicate an abnormally low cell temperature. Indeed, they were quite within the range noted with normal rats.<sup>9</sup> The low basal metabolism of the larger rats, and particularly the enormously large rats, is therefore not due to a low cell temperature.

The number of basal metabolism measurements on the "giant" rat reported in the literature is small. The interest in these animals centers chiefly around the effect of growth-promoting extracts of the anterior pituitary upon the basal metabolism. Lee (9) found that with 4 rats (sex not stated), ranging in weight from 336 to 512 grams, the average heat production in 16 determinations over a period of 5 weeks was 697 calories per square meter of body surface per 24 hours, with a standard deviation of  $\pm 21$  calories. These animals had been injected with the extract. Control rats (females) were selected of the greatest size possible and ranged in weight from 200 to 270 grams. Nine determinations with these rats showed

<sup>8</sup> These *actually measured* values for the basal metabolism of these male rats on the surface-area basis are all appreciably lower than the average value of 800 calories *suggested* by Benedict and MacLeod (*Journal of Nutrition*, 1929, 1, 392) as the basal metabolism of the male rat at thermic neutrality.

<sup>9</sup> For the literature on the body temperature of the normal rat see S. V. Gudjonsson, *Jour. Physiol.*, 1932, 74, 73.

a heat production of 805 calories, with a standard deviation of  $\pm 50$  calories, which Lee states agrees well with the average of a large number of determinations on other normal rats under the same conditions (measured at  $29.5^{\circ}$  C.). "Giant" rats produced by hormone feeding may possibly be considered to be wholly unphysiological, but so far as the evidence is concerned it shows that the "giant" rats produced with growth-promoting extracts had a lower heat production than the smaller rats.

#### GASEOUS METABOLISM AND HEAT PRODUCTION OF UNUSUALLY LARGE RATS DURING PROLONGED FASTING

The gaseous metabolism of the two very large rats, Nos. 25 and 26, was determined during their prolonged fasts every 3 or 4 days. Owing to the seemingly important changes in the type of metabolism of rat No. 26, this animal was studied every other day toward the end of the fast. The results of these metabolism observations, together with the rectal temperatures noted on the same days, are given in Table III.

In general one can say that there is no pronounced difference between the rectal temperatures of these very large rats and the body temperatures commonly reported for the rat of normal size. With neither animal was there any significant change in rectal temperature due to fasting. It would appear, therefore, that at an environmental temperature of  $27^{\circ}$ , and particularly after a stay of several hours at  $30^{\circ}$ , the rectal temperatures of these very large fasting rats, even after they had lost more than half their initial weight, were essentially normal.

With rat No. 26 from the 29th day until the end of the fast on the 56th day, the total oxygen consumption per 6 hours was fairly constant, although the body weight was decreasing rapidly during this time. Indeed, after the first 20 days, during which there was a rapid decrease in oxygen consumption, the metabolism of the organism as a whole can be considered to have remained essentially unchanged. At the end of the fast, the total oxygen consumption was only about 8 per cent less than it was on the 22nd and the 26th days. Still, the total oxygen consumption had been somewhat lower between the 29th and the 46th days of the fast, the average value being about 19 per cent less than the total oxygen consumption on the 22nd and the 26th days. Rat No. 25 did not fast as long, but here again beginning at about the 22nd day the metabolism of the organism as a whole remained constant until the 38th day, in spite of the fact that there was a considerable loss in body weight during this time. With rat No. 25 the level of the oxygen consumption at the beginning of the fast was 2751 cc., although this was definitely higher than that measured

TABLE III  
METABOLISM OF UNUSUALLY LARGE MALE RATS DURING PROLONGED FASTING  
(Living at 27° C.; measured at 30° C.)

Rat No. and duration of fast	Body weight	Rectal temp.	Oxygen consumed per 6 hours	Heat production per 24 hours		Activity
				Per kg.	Per sq. m.	
days	gm.	°C.	cc.	cal.	cal.	%
Rat No. 26*						
1	797	37.4	2505	59.1	603	—
5	748	37.9	2189	55.1	549	10
9	716	37.3	2002	52.7	517	9
12	688	37.3	1751	47.8	464	8
15	665	38.0	1713	48.4	465	10
19	634	37.8	1722	51.1	482	11
22	612	37.8	1552	47.7	445	11
26	588	37.7	1624	51.9	478	12
29	566	37.0	1248	41.5	377	5
33	544	37.8	1618	55.9	502	7
36	516	37.4	1268	46.3	408	4
40	494	37.3	1466	55.9	485	8
43	476	36.0	1124	44.3	381	4
46	452	36.8	1187	49.3	417	6
48	433	37.3	1400	60.7	506	9
50	427	37.4	1432	63.0	522	6
52	410	37.6	1470	67.3	551	9
54	397	37.1	1441	68.3	551	6
56	382	37.4	1434	70.7	563	11
Rat No. 25*						
1	706	37.4	2751	73.2	717	—
3	685	38.1	2363	64.8	628	—
8	637	37.8	2530	74.7	706	—
10	623	37.6	2252	68.1	638	—
15	580	37.4	1974	64.0	586	—
17	562	37.8	1965	65.8	596	—
22	524	37.9	1655	59.4	527	—
24	512	38.1	1840	67.6	595	13
29	473	37.8	1635	65.1	557	7
32	440	37.9	1709	73.0	611	10
35	422	37.3	1612	71.8	592	10
38	398	37.8	1653	78.1	632	17

\* Rat No. 26 was 430 days old at the start of the fast; the age of rat No. 25 was not known but it was probably in its second year.

a few days before under the usual basal conditions. From the 22nd to the 38th day of fasting the oxygen consumption averaged 1684 cc., or 61 per cent of that at the start. At the beginning of the fast the oxygen consump-

tion of rat No. 26 was 2505 cc. per 6 hours. From the 26th day on it averaged 1393 cc., or 56 per cent of that at the start of the fast.

The results for the total oxygen consumption of these unusually large fasting rats are in striking contrast with the values for fasting female rats studied earlier (10). Thus, female rats fasting 16, 15, and 24 days, respectively, had an oxygen consumption (at 28°) in all cases at the end of the fast less than 50 per cent of the initial value. With the very large rats, Nos. 25 and 26, the metabolism at the end of the fast was 61 and 56 per cent of the initial level. The female rats weighed on the average 210 grams at the start of the fasts. The very large rats weighed three and one-half times as much. Since the 3 female rats died when the oxygen consumption had fallen to approximately half of what it was at the start of the fast, one would not have expected the very large rats to die for some time after 56 and 38 days. It is regretted that rat No. 26 was killed and that the realimentation experiment was inadvisably carried out with rat No. 25. It is obvious from these comparisons alone that these unusually large rats were amply provided with reserves to withstand a very long fast. At the end of 40 to 50 days of fasting their organisms still had a high metabolism. At the end of their fasts the female rats weighed on the average 114 grams, and the oxygen consumption averaged 514 cc. per 6 hours. The average weight of the two very large rats at the end of their fasts was 390 grams, or over 3 times that of the female rats, and the average oxygen consumption was 3 times greater. On this basis one might expect that the very large rats were approaching death. From the autopsy records, however, it is clear that they had still an abundant reserve.

Since the very large rats were losing weight continually during the fast and the oxygen consumption remained at a fairly constant level, it is not surprising to find that the heat production per kilogram of body weight definitely increased toward the end of the fast. In this respect the very large rats differ again from the female rats, the heat production of which per kilogram of body weight remained essentially constant after the first one or two days of fasting. With rat No. 26 the heat production per unit of weight decreased markedly until the 12th day. It reached a minimum on the 29th day and then returned to a value even higher than it was at the beginning of the fast. With rat No. 25 the values are more variable, probably due to the activity, which was not recorded in the first 7 experiments. As this rat had not been confined in a small cage for a very long time, it was probably less content in the respiration chamber than was rat No. 26. The minimum value with rat No. 25 was found on the 22nd day and the maximum value on the 38th day. The longest fast with the female



rats was only 24 days, but the uniformity in their heat production per kilogram of body weight after the first few days of fasting is in striking contrast to the increases and the variability noted with the very large rats.

Rubner has pointed out that the heat production per unit of surface area does not follow the "surface-area law" in fasting animals, but that there is a pronounced decrease in the heat production on this basis during fasting. This decrease was shown to some extent by both of our very large rats, but not throughout the entire fast. Thus, the heat production per square meter of body surface per 24 hours, in the case of rat No. 26, *decreased* as the fast progressed until the 29th day, when it reached a minimum. Toward the end of the fast, however, it *increased* to a level as high as it was on the 5th day. With rat No. 25 there was a decrease in the heat production per square meter of surface area during the first 15 days of the fast. The day-to-day variability in the heat production of this rat, however, makes it difficult to estimate the real change in metabolism. The heat production per unit of surface area observed on the first day of prolonged fasting was unusually high, 717 calories. The basal metabolism of this same rat measured 12 days before the long fast (see Table II) was only 621 calories. Again on the 8th day of the prolonged fast a high heat value was observed, 706 calories, as compared with 628 calories on the 3d day and 638 calories on the 10th day. From the 15th to the 29th day the heat production was slightly lower, with a variability up and down from a minimum of 527 calories on the 22nd day to a maximum of 595 calories on the 24th day. From the 32nd to the 38th day of the fast the heat production was somewhat higher, 592 to 632 calories per square meter of surface area. Here toward the end of the fast there is no indication of a consistent decrease in the heat production per unit of surface area. In contrast to these results, the heat production of female rats was found to decrease fairly regularly throughout the course of fasting (10).

Comparison of our two very large rats shows that in general rat No. 25 had a higher metabolism than rat No. 26. If the two rats are compared only during the first 38 days of fasting, one sees that the average heat production of rat No. 26 was measurably under 500 calories per square meter of body surface, whereas that of rat No. 25 was almost 600 calories. It is clear that we were dealing here with definite individuality in the two animals.

The body surface of the very large rats was computed from each individual body weight obtained during the progress of the fast. This immediately brings up the question as to what is the surface area of a fasting rat. The surface area of a normal rat is usually considered to be its skin area. The skin area is determined with difficulty, owing to the danger of

stretching the skin, but many writers have assumed that with normal rats the surface area and the skin area are readily computed from the two-thirds power of the body weight times the constant 9.1. The skin area as the fast progresses must automatically become much larger than the surface area of the body. Indeed, if the heat production of these fasting rats were calculated per unit of skin area, the results might be considerably lower than those recorded in Table III, possibly as low as 300 calories per square meter of skin area. It is inconceivable, however, that the skin area of a fasting rat weighing half as much as it did at the start of the fast would even approximate half the initial skin area. It is furthermore inconceivable that the computation of the skin area as such can be made by applying the constant 9.1 to the two-thirds power of the body weight of the emaciated rat. At the end of the fast the skin of rat No. 26 had not shrunk to correspond to its lower weight. The skin was wrinkled and hung in folds and was probably much the same in area as at the start of the fast. On the other hand, if we are dealing with surface area in contradistinction to skin area, it is not impossible to assume that at least an approximation of the surface area may be obtained by the formula  $S = 9.1 \times w^{2/3}$ . For want of a better method of expression, this formula has been used in our calculations of the heat production per square meter of surface area during the fasting periods. Precisely the same procedure was carried out in the earlier studies with fasting female rats.

It is clear from inspection of the values for the total oxygen consumption and the heat production per unit of weight and of surface area that the metabolism of our two very large rats during the latter parts of their fasts was definitely increased. Activity cannot account for the increase. Since the rectal temperatures were normal, there could not have been an increased cell temperature to account for it. As was pointed out in the analysis of the losses in body weight during the fasts, an increased protein disintegration must be thought of, but unfortunately the nitrogen in the excreta was not determined. Comparison of the very large fasting male rats with the fasting female rats of normal size makes it seem probable that the very large rat is an altogether different organism from the normal-sized rat and that its heat loss is regulated by different laws. It serves admirably to illustrate how deficient is our knowledge of the physiology of fasting, in spite of the extensive research with both man and animals carried out in the past few decades. The fact that it is possible for a rat to grow to such a huge size and show no abnormality in configuration or general condition, and yet have a metabolism so different from that of

rats of normal size, suggests lines of study that must be carried out in the near future.

### SUMMARY

Two unusually large male rats (maximum weights 830 and 766 grams) fasted 57 and 38 days, respectively, when one was killed for autopsy and the other was refed. In neither instance did the animal appear moribund. At the end of the fasts the body weights were 46 and 55 per cent of the initial weights (822 and 730 grams). With both rats the daily loss in weight remained relatively constant, after an initial pronounced decrease, until toward the end of the fast when the drafts upon body weight were heavier. The autopsy of the rat that fasted 57 days showed it still had a liberal supply of fat.

In a comparison of the *basal* metabolism of the very large rats with smaller rats, it was found that the metabolism was lower per unit of weight and per unit of surface area, the larger the rat. Male rats weighing from 307 to 396 grams produced on the average 92 calories per kilogram of body weight and 708 calories per square meter of body surface per 24 hours. Male rats weighing from 407 to 562 grams produced 77 calories and 664 calories, respectively. The two unusually large rats produced 59 and 63 calories per unit of weight and 603 and 621 calories per unit of surface area. The rectal temperatures of the very large rats ( $37.4^{\circ}$ ) were similar to the body temperatures of the medium-sized rats ( $37.6^{\circ}$ ) and the large-sized rats ( $37.5^{\circ}\text{C.}$ ).

During the prolonged fasts the total oxygen consumption decreased until about the 20th day of the fasts, but thereafter remained relatively constant, although the rats were losing weight rapidly. The heat production per kilogram of body weight and per square meter of body surface decreased markedly during the first few days, but toward the end of the fasts definitely increased. This is in striking contrast to the reaction shown by fasting female rats, whose heat production per unit of weight was essentially constant after the first one or two days, and per unit of surface area decreased as the fast progressed. With the two very large rats there was no increase in cell temperature or increase in activity to account for the increased metabolism toward the end of the fasts. Indeed, the rectal temperatures during the prolonged fasts were not different from those of rats under normal conditions. These findings suggest that there was an increased protein disintegration toward the end of the fasts, although the nitrogen in the excreta was not determined.

With one of the very large rats minimum values of 377 and 381 calories

per square meter of body surface per 24 hours were recorded on the 29th and the 43d days of fasting. This is probably the lowest heat production that has been noted with warm-blooded animals having a rectal temperature of essentially 37°C.

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# CALCIUM AND PHOSPHORUS OF SALIVA IN RELATION TO DENTAL CARIES\*

By

REBECCA B. HUBBELL AND RUSSELL W. BUNTING

*(From the Department of Physiological Chemistry, School of Medicine and  
Department of Oral Pathology, School of Dentistry, University of Michigan,  
Ann Arbor)*

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THERE have been numerous attempts to show a relation between dental caries and the amount of calcium in the saliva. Fewer studies have considered the phosphorus content as well. Some of those who believe that the preservation of the teeth is somewhat dependent on the chemical composition of the surrounding fluid, associate the absence of decay with a relatively high salivary calcium. Bunting and Rickert (1), who determined the calcium by weighing the ignited oxide, found that when the calcium was high the enamel was dense and opalescent, while when it was low the teeth were white or blue white and had the appearance of permeability or roughness. Later work from the same laboratory (2) confirmed these findings and stated that high salivary calcium was associated with immunity to caries. In partial confirmation of these results, Horton, Mar-rack, and Price (3), after examining salivas of several hundred children with varying numbers of carious teeth, report that as the number of carious teeth increases, the calcium of the saliva decreases. However, they report higher salivary calcium in the early stages of caries development, which leads them to conclude that the change in calcium content is not the primary change.

On the other hand, some workers, such as Spencer-Payne (4), Clark and Levine (5), and Leonard (6), have been unable to find such relations. Clark and Levine (5), in a study of various inorganic constituents of saliva emphasize particularly that the calcium content of individual salivas varies from time to time, so that it is difficult to detect any relation which may exist between salivary calcium and the condition of the teeth. This view was confirmed by Leonard (6). More recently, Karshan, Krasnow, and Krejci (7) have made a study of salivas from a group of dental students and have found slightly higher calcium for the immunes, but "the difference is not so great as some others have reported."

In the few studies concerned with the phosphorus of the saliva there has

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been little agreement. Hawkins (8) found it lower for caries free individuals than for those who were susceptible to caries, while Karshan, Krasnow and Krejci (7) found it higher. Entin and Geikin (9) and Horton, Marrack and Price (3) could find no correlation between phosphorus of saliva and caries.

We have studied the salivas of school children from seven to sixteen years of age. This was done to secure more data on the calcium and phosphorus of the saliva in relation to caries and at the same time to determine whether there would be changes in tooth condition following slight dietary improvement and whether such changes would be reflected in the salivary calcium and phosphorus. The children studied were divided into five groups:

*Group I* included twenty-five children with active caries. The home diet was supplemented each school day by one quart of milk, two ounces of tomato juice and eight drops of viosterol.<sup>1</sup> The milk was given in three portions, one half pint at the opening and at the close of school, and a pint at the noon lunch hour. The tomato juice and viosterol were mixed and given with the noon meal.

*Group II* included twenty-five children with active caries. These received in addition to the home diet one quart of milk and two ounces of tomato juice, but no viosterol.

*Group III* was a control group of thirty children with active caries and the home diet was not supplemented.

*Group IV* was a control group of ten children with no active caries and in most cases no fillings. The members of this group received the same supplements that were given to those in Group I.

*Group V* included twelve children with no active caries and in most cases no fillings. This also was a control group and no supplement was made to the home diet.

Dental examinations<sup>2</sup> were made four times, at the beginning and at the end of, and twice during the period of the investigation. Before any of the children received supplementary feeding there were at least three analyses of saliva for each child (Table I). Groups I, II and IV received the supplements indicated beginning Jan. 10, 1930. These were discontinued June 1, resumed with the opening of school Sept. 15 and discontinued completely

<sup>1</sup> The viosterol was furnished through the courtesy of the Abbott Laboratories. The product first used was that known as Viosterol in Oil 100 D. Later Viosterol in Oil 250 D was used when this change was authorized by the Council on Pharmacy and Chemistry of the American Medical Association.

<sup>2</sup> The authors acknowledge with pleasure their indebtedness to Dr. Dorothy G. Hard and Dr. Philip Jay for assistance with the dental examinations.

TABLE I  
SUMMARY OF CALCIUM AND PHOSPHORUS CHANGES  
All results are expressed in mg. per 100 cc. of saliva.

Group	Number of subjects	Number of samples	Calcium				Phosphorus			
			Range		Average		Range		Average	
			1	2	1	2	1	2	1	2
		1* 2**			mg.	mg.	mg.	mg.	mg.	mg.
Ia b	11	35	2.8-7.2	2.6-7.2	4.8	5.0	10.4-15.6	10.3-16.0	12.9	13.2
	8	25	3.4-6.8	2.6-7.0	5.0	5.3	9.4-15.3	9.8-15.9	12.5	12.7
IIa b	12	35	2.5-7.3	2.2-8.0	4.6	5.0	9.8-15.9	9.4-15.7	12.0	12.3
	5	16	2.8-6.6	3.4-7.3	4.7	5.1	10.0-15.2	9.1-15.4	13.4	13.2
IIIa b	11	34	3.4-7.4	3.5-7.5	5.3	5.8	9.7-17.0	10.4-16.8	13.1	13.4
	12	36	2.6-6.4	2.8-7.4	4.9	5.2	9.8-16.3	10.1-16.8	12.8	13.1
IVa b	4	12	3.2-6.8	2.8-6.4	4.8	5.1	9.6-16.1	9.6-15.6	13.3	13.7
	1	3	4.4-6.2	4.5-7.3	5.5	5.8	10.6-11.1	10.1-13.0	11.0	11.7
Va b	9	28	4.0-7.3	3.6-7.6	5.2	5.6	9.7-16.5	9.8-16.6	12.6	12.7
	2	6	4.2-7.3	4.4-7.5	5.4	6.1	11.7-15.3	10.6-15.4	13.2	13.3

\* Period 1 represents three analyses for each child.

\*\* Period 2 represents eight analyses for each child, made during the months when dietary supplements were given.



April 1, 1931. During this time salivary analyses were made for each child once every six weeks, with the exception of summer. This gave at least eight determinations for each individual during the period.

The saliva used was paraffin activated. The samples were obtained at ten a.m. in all cases. The saliva was transferred through a short-stem glass funnel into a small flask. Collecting was continued for 15 to 30 minutes, depending on the rate of flow. The samples were analyzed for total solids, ash, calcium and phosphorus. Two ten cc. portions were transferred to 40 cc. platinum dishes and dried in an electric oven over night to determine total solids. The dried material was ashed in an electric muffle just below dull red heat and the residue was weighed. The ash was dissolved by digesting it with 1 cc. portions of hot 1:3 hydrochloric acid and the solution was transferred to a 25 cc. volumetric flask. Five cc. portions of this acid ash solution were used for the determination of calcium by the Clark and Collip (10) modification of the Kramer and Tisdall (11) method, and 0.5 to 1 cc. portions for the determination of phosphorus by the method of Fiske and Subbarow (12).

### DISCUSSION

In summarizing the data obtained, each of the five groups has been considered in two periods. The first covers the analyses made during October, November and December 1929, before any of the children had received dietary supplements. The second division includes the analyses from January 1930 through March 1931 when an attempt was made to improve the diet for selected groups.

On the basis of the dental examination made at the conclusion of the work, each of the five groups was divided into two sub-groups, *a* and *b*. Sub-group *a* in each case includes those individuals who developed no new cavities during the time under observation, while in sub-group *b* are placed those showing new lesions or extensions of old ones. Table I summarizes data for all individuals for period 1 and period 2.

For period 1 there was no distinct difference either in range or average for the calcium or phosphorus of the saliva of children with active caries as compared with those who were caries free. Furthermore, we found rather wide variations in the calcium and phosphorus content of samples from the same individual at different times. This is shown in Table II which gives calcium and phosphorus content of salivas of six caries-susceptible and six caries-free children for the three determinations during this preliminary period. For the second and longer period, when selected individuals received supplements to the home diet, Table I shows that in Group I, re-

ceiving milk, tomato juice and viosterol, 58 per cent of those who were under observation for the whole time showed an arrest in caries activity. For Group II, in which milk and tomato juice were the only supplements, 70 per cent showed improved clinical condition, while for Group III, with

TABLE II  
TYPICAL CALCIUM AND PHOSPHORUS VALUES FOR SALIVA OF CARIES-SUSCEPTIBLE  
AND CARIES-FREE CHILDREN

All results are expressed in mg. per 100 cc. of saliva.

Caries-Susceptible		Caries-Free	
Calcium	Phosphorus	Calcium	Phosphorus
mg.	mg.	mg.	mg.
L.A. 3.2	10.4	D.H. 5.5	14.0
4.4	9.8	4.9	12.5
5.6	10.7	6.8	13.2
M.B. 2.9	10.4	C.L. 5.0	15.1
5.3	11.6	4.4	13.0
5.1	8.5	5.2	13.8
L.H. 4.1	14.8	F.S. 4.2	12.3
6.1	13.3	2.2	11.7
5.1	14.7	3.2	13.4
A.K. 2.2	10.4	L.S. 4.6	9.7
4.3	11.5	6.8	10.0
2.1	10.3	4.7	10.5
R.S. 5.1	10.5	M.W. 5.0	10.7
7.3	10.9	4.5	9.8
7.7	12.0	4.3	10.0
R.K. 4.9	13.4	C.Y. 4.7	13.1
2.8	15.0	5.4	13.3
3.5	15.7	5.3	14.1

no supplement, 48 per cent developed no new caries. Eighty-one per cent of those in Group IV and Group V were caries free. However, the number of subjects in these two groups was so small that adequate comparisons cannot be made. Thus, there was a somewhat lower incidence of caries corresponding with dietary supplement, but the differences were not marked.

The calcium and phosphorus figures show even less change. While most of the values are somewhat higher in period 2 there is no correlation between these changes and either the diet or the arrest of caries. Moreover,

the averages of all values for the second period agree very closely with the average figures which have been obtained in this laboratory for all children, regardless of diet. A total of 902 determinations on children with active caries has given an average calcium of 5.1 and phosphorus of 12.8 mg. per 100 cc., while 275 determinations on children who were free from caries have averaged 5.4 calcium and 13.3 mg. phosphorus.<sup>3</sup>

While we could detect no constancy of calcium or phosphorus content of saliva and no consistent variation with changes in tooth condition or with the diet supplements used, it has become evident that the volume of saliva secreted in a given time should be considered. The average rate of flow for these children was 1 cc. per minute. There were, however, many individuals who constantly produced much more than this, and others whose rate was much lower. If we consider two extremes in rate, 0.5 and 2 cc. per minute, we find average figures as shown in Table III. The lower values for

TABLE III  
VARIATION OF CALCIUM AND PHOSPHORUS OF SALIVA WITH RATE OF SECRETION

Volume	Number of samples	Calcium	Phosphorus	Calcium	Phosphorus	Total Solids	Ash
cc./min.		mg./100 cc.		mg./min.		gm./100 cc.	
0.5	65	6.0	15.0	0.030	0.075	0.650	0.182
2.0	97	4.8	11.9	0.096	0.238	0.541	0.156

calcium and phosphorus with the larger volume are not due entirely to dilution, for the figures are not proportional to the volumes. If we calculate the total amount of salivary calcium and phosphorus per minute for each group we find that those with the more rapid flow secrete about three times as much as the slower. When the calcium and phosphorus are expressed in mg. per 100 cc. of saliva much higher values are reported for those individuals with the slower rate of secretion. If the saliva is a possible factor in the determination of immunity of susceptibility to caries, it seems as if one should consider not only the relative amounts of the constituents as expressed in the usual way, but should take into account as well the total amount of material available at a given time. At present we can make no satisfactory correlation between rate of flow and tooth preservation, although there appears to be a slight tendency for the caries-free individuals to secrete larger volumes than is the case of those with active caries.

<sup>3</sup> These figures include some unpublished data.

## SUMMARY

1. In a group of children from seven to sixteen years of age there was no relation between calcium and phosphorus content of the saliva and the occurrence of dental caries.

2. When the diet was supplemented by the daily addition of one quart of milk and two ounces of tomato juice, with or without viosterol, there was a slight tendency toward a decrease in the incidence of dental caries. This improvement in tooth condition was not accompanied by any consistent change in the salivary calcium and phosphorus.

3. Evidence has been presented that the volume of saliva secreted in a unit time should be considered in interpreting salivary analyses.

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